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Award Number: DAMD17-02-1-0107

TITLE: Formulated delivery of enzyme/prodrug and cytokine gene therapy to promote immune reduction of treated and remote tumors in mouse models of prostate cancer

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REPORT DATE: January 2007

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2007	Final Addendum	01 Jan 06 – 31 Dec 06

4. TITLE AND SUBTITLE
Formulated delivery of enzyme/prodrug and cytokine gene therapy to promote immune reduction of treated and remote tumors in mouse models of prostate cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0107

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of New South Wales
Sydney NSW 2052 Australia

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Prostate cancer is the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery or radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. We use gene therapy, alone and in combination with hormones called cytokines that stimulate the immune system. The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations. In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT)) is delivered to a cancer cell by a virus, or expressed by molecular engineering, so that harmless bacterial proteins are made. When followed by a pro-drug, 5 fluorocytosine (5FC), cancer cells that make CD/UPRT convert 5FC to a toxin that kills the original and neighbouring cells. This system works in slow growing tumors like prostate cancer. Killing the tumor cells attracts immune cells. We are identifying these and then delivering cytokine genes that attract more immune cells into the tumors. We will deliver the cytokine gene alone or with the suicide gene because in other studies, combination therapy works better.

15. SUBJECT TERMS
Gene-directed enzyme prodrug therapy, mouse model, TRAMP, RM-1

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	127	**19b. TELEPHONE NUMBER** *(include area code)*

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Formulated Delivery of Enzyme/Prodrug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

Final Report, January, 2007. DAMD17-02-1-0107

INTRODUCTION:

Prostate cancer (PC) is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatment strategies are needed. The **subject** of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. These therapeutic genes are delivered using lentiviral or adenoviral vectors, or by stable transfection into prostate cancer cells. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5FC), is then given, cancer cells producing CDUPRT convert 5FC to a toxin that kills the original cell and others nearby. This strategy is suitable for slow growing tumors like PC. Killing the tumor cells attracts immune cells. The **scope** of the work involves preparation of the gene vectors, optimizing the conditions required for delivering the genes of interest by transfection or by using viral vectors, and identification of the immune cells that infiltrate the tumor when gene therapy is used. We are then using cytokine genes delivered into the tumor to attract more immune cells to this site. We have compared the effects of delivering the cytokine gene therapy alone, the suicide gene therapy alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of tumor cells that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with PC.

BODY:

New cell lines have had to be prepared for the studies described below. In addition, we have had to prepare and characterize both plasmids, adenoviral and lentiviral vectors containing the genes of interest for delivery into PC. Not only have we manufactured the plasmids and recombinant viral vectors for delivery of the genes of interest, but we have also prepared stable transfectants from a murine PC cell line, the RM1 line, kindly provided by Dr T Thompson, Baylor College, Texas. The use of transfected cell lines has allowed us to generate a maximum effect *in vivo*, so that we could compare the possibilities that could be generated using a viral vector as the delivery vessel. In the first instance, the work was based on our previous studies that showed that the gene, purine nucleoside phosphorylase (PNP), could be used for gene-directed enzyme prodrug therapy (GDEPT) directed against PC (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.,* 2002; Voeks *et al*., 2002a). However, due to problems with intellectual property, we were no longer able to use this gene, and moreover, workers from CSIRO decided not to be involved in the ongoing work. Instead, we recruited Dr Aparajita Khatri, PhD (starting August, 2003) Dr Bing Zhang, PhD (started November, 2003, but left June 2004) and Ms Eboney Doherty, Bsc. Hons, who started in July, 2003. Unfortunately, Dr Zhang left us after 6 months for unforseen family reasons, and Ms Doherty left to study medicine. We subsequently recruited Ms Jane Chapman for technical help.

She started work in April, 2004). We then recruited Dr Yasmin Husaini to continue the work. She left at the end of 2006 as we had run out of money. Instead of using PNP, we have chosen the fusion gene, CDUPRT for the following reasons: CD converts 5 fluorocytosine to 5 fluorouracil, whose metabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) damage DNA and RNA respectively. The rate-limiting step in the generation of 5FdUMP and 5FUTP is the formation of an intermediary metabolite, 5-fluorouridine mono-phosphate (5FUMP). 5-FUMP is only produced after a series of catalysed enzymatic reactions. This can be circumvented by the ability of UPRT to convert 5FU directly to 5FUMP thereby leading to more efficient production of anti-tumor metabolites, 5FdUMP and 5FUTP (Tiraby *et al*., 1998). UPRT sensitizes cancer cells to low doses of 5FU (Kanai *et al*., 1998), and when used in conjunction with CD and 5FC in GDEPT, was more effective than CD-GDEPT alone against colon cancer (Koyama *et al*., 2000; Chung-Faye *et al*., 2001) and glioma (Adachi *et al*., 2000) *in vitro* and *in vivo*. There are very limited reports of this combination (CDUPRT) being used against PC. Thus *drugs generated by CDUPRT can kill both dividing and non-dividing cells*. This is important in PC, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.,* 2000; Pierrefite-Carle *et al.,* 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al*., 1999).

In the first two years of this project, we made lentiviruses expressing CDUPRT, but experienced difficulty in making sufficient virus for *in vivo* experiments. **We therefore implanted RM1 PC cells stably transfected with CDUPRT in the prostate of C57BL/6 males, and treated the mice with 5FC to test proof of principle of the CDUPRT/5FC GDEPT in our model system.** Last year, we reported on the use of CDUPRT RM1 cells in the presence and absence of plasmids expressing mIL12 and mIL18, either singly or in combination in order to assess whether immunotherapy is synergistic with GDEPT. In this final report, we detail our findings from similar experiments in which we used adenoviral vectors expressing mIL12 or mIL18 or both together instead of plasmids. The results are shown in the poster which is attached.

DOD Alternate: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. The new program Statement of Work, accepted by the DOD, was shown in Appendix 1 of our Annual Report for 2003 (January, 2004). New staff were recruited, as described above, and because of this, the work could not be commenced until July, 2003. Unfortunately, we again lost staff for various reasons, and had to begin recruitment again. The current report represents a total of 3 years of work.

Task 1:
GDEPT alone. Assess the ability of lentivirus expressing GDEPT (based on the fusion gene, cytosine deaminase/uracil phosphoribosyltransferase (CD/UPRT) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 1-12)
Methods and results were shown in the 2006 report.

Whilst we tried to optimize conditions in order to procure sufficient virus to conduct our *in vivo* experiments, we eventually went via a different path, as this proved too difficult and slowed the work down too much.

TASK 1b. Establish conditions for implanting TRAMP-C1 cells subcutaneously in transgenic TRAMP mice.

A relevant preclinical model that represents all stages of the human prostate cancer (PC) is required to evaluate potential therapies. Although commonly used, transplantable syngenic or xenogenic murine models do not emulate the considerable biological and technical challenges inherent in cancer treatment. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model closely mimics early stages of the human disease. However, this model does not adequately represent late stage androgen-independent, metastatic cancer, and the timing to cancer is slow. We have therefore been working with TRAMP cell lines to circumvent this problem. As described in the 2005 report, we established that implanted TRAMP-C1 tumor cells in TRAMP (transgenic adenocarcinoma mouse prostate) mice did not grow until after 2 months, when they formed poorly differentiated prostate cancers, with loss of glandular architecture (Jan 2004 report, Appendix II, **Figure 3**).

Development of the TRAMP mouse model to represent different stages of prostate cancer

In order to conduct these experiments in TRAMP transgenics, the model has to be fairly consistent. We are currently breeding these mice at Biological Resource Centre, UNSW, available to us. However, recently we have faced a number of problems. The mice did not breed well (litter size has dropped to between 2-4), compromising the number of TRAMP positive male mice available (25% of a litter). Further, we found that the mice did not develop tumors at the time anticipated (~28-30 wks) with a fall in incidence to <40%. These problems further enhanced the already difficult logistics of conducting such complex experiments in a transgenic model. In addition, although reported by others (Greenberg *et al*., 1995; Gingrich *et al*., 1996) we have failed to find consistent metastases in these mice, and those we've seen have been limited to rare lymph node metastasis (Voeks *et al*, 2002b). Hence, to improve the logistics of experimental work and to broaden the model to represent late stage PC we have derived hormone refractory prostate cancer (HRPC) cell lines and characterized them *in vitro* and *in vivo* after implantation in syngeneic TRAMP null and C57BL/6 mice. The growth of TRAMP tumor derived parentals in the immunocompetent TRAMP null and C57BL/6 mice is well characterised in our laboratory (Voeks *et al*., 2002b). The experimental description and the results from this study are reported below.

Characterization of HRPC TRAMP cell lines:

Please see draft paper attached below, for submission to Prostate. (Appendix II)

This section of the work has been performed by PhD student, Varinder Jeet.

TASK 1c Optimize dose of virus needed to establish GDEPT in orthotopically implanted RM-1 tumors when formulated with plasmid.

Instead of using lentivirus to express CDUPRT, we used the stably transfected RM1 cells (see below). Therefore we did not perform Task 1c. We had previously shown (see 2006 report) that C57BL/6 mice could tolerate 5FC at a dose up to 500 mg/kg/mouse, given intraperitoneally (ip) every day for 13 days without systemic toxicity as observed by serum analysis (See January 2004 report, Table 1) or by histological examination of major organs (January 2004 report, Appendix II, **Figure 4**).

We used stably transfected RM-1 cells expressing green fluorescence protein (GFP) and CDUPRT (test) (known as RM1-GFP/CDUPRT) or GFP alone or GFP.LacZ (controls) (known as RM1-GFP or RM1-GFP/LacZ) as described in our January 2004 report, section c. Methods were developed to measure CDUPRT expression *in vitro* by testing the capability of the cell/ lysates to catabolize the prodrug 5FC to 5FU (by HPLC: Jan 2005 report, Appendix II, **Figure 5A**) and by examining the effects of CDUPRT expression in the presence of 5FC on cell proliferation (Jan 2004 report, Appendix 2, **Figure 7**). Experiments confirmed that homogenates from RM1-GFP/CDUPRT

tumors grown *in vivo* expressed CDUPRT enzymic activity by the HPLC assay. The sensitivity of the HPLC-based assay was also evaluated and the minimum protein concentration at which the 5FC-5FU conversion was detectable by HPLC was *0.3 mg,* equivalent to *~2x10^6* cells *in vitro*.

TASK 1d. Assess ability of optimal doses of CD/UPRT-GDEPT (and control plasmid) injected intraprostatically into RM-1 tumors together with systemic pro-drug (5 fluorocytosine, 5FC) treatment to suppress local prostate and metastatic (lung) tumor development. &
TASK 1e. Examine other tissues for signs of toxicity that might result from escape of the CD/UPRT GDEPT virus from the site of injection.

Again, as viruses were not used to induce CDUPRT expression in vivo, we implanted RM1 cells stably transfected with CDUPRT in the prostate and treated the mice with 5FC to test **proof of principle**. The data acquired are reported in our paper, Khatri A, Zhang B, Doherty E, Chapman J, Ow K, Pwint H, Martiniello-Wilks R, Russell PJ. Combination of cytosine deaminase with uracil phosphoribosyl transferase leads to local and distant bystander effects against prostate-cancer in C57BL/6 mice. *J Gene Medicine*, 2006;8(9):1086-96. (Appendix II). We have demonstrated proof of principle that CDUPRT-GDEPT + 5FC kills RM1 prostate cancer cells when given into the prostate, and also inhibits the growth of pseudometastases in the lung. Both a local and a distant bystander effect occur *in vivo*.

TASK 1f. Identify using immunohistochemistry, the immune cell types infiltrating the prostate tumors.
These studies are also described in the above paper, Khatri et al, J Gene Med (Appendix II). In summary, immune cells infiltrating the primary tumor include CD4 T cells, F480 positive macrophages and AsialoGM1+ NK cells. In addition, there was a decrease in the vasculature to the tumor (CD31 positive cells) and an increase in apoptosis as measured by Tunel assay.

Task 2.
pCytokine work: Assess the ability of lipid-enhanced delivery of an murine IL-12 or IL-18 expressing plasmids (pCytokine) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

This was described in detail in our 2006 report.

The following studies have not yet been undertaken, but instead, work has been done using RM1-GFP/CDUPRT cells implanted into the prostate of C57BL/6 mice. In the 2006 report, we described the use of these cells together with mIL12 and mIL18 plasmids injected introtumorally; mice were treated with 5FC to elicit GDEPT or saline, ip, daily until euthanasia (please see below).In this report, (2007), we have repeated these studies using adenoviruses expressing mIL12 and mIL18 instead of the plasmids. The results are shown in the poster for the 2007 AACR conference which is occurring in April, 2007 (Appendix II).

The use of either AdmIL12 or AdmIL18 delivered intraprostatically into RM1 parental tumors did not have any significant effect on local prostate tumor growth, but caused a substantial reduction in the number of lung colonies after iv injection of RM1 cells in the treated mice. This suggested that systemic immune responses were stimulated.

TASK 3. **Combination therapy**: Assess the ability of delivery of a combined virus borne GDEPT and lipid delivered plasmid-borne cytokine gene therapy to suppress orthotopic and metastatic prostate cancer in RM-1 model and in TRAMP mice carrying sc TRAMP-C1 grafts. (Months 22-33)

a. Determine whether pCytokine-enhanced immune activity affects GDEPT.
b. Determine the effects of injecting lentivirus expressed GDEPT and pCytokine intraprostatically (using optimal doses of each component as revealed by Tasks 1A and 1B) on orthotopic tumor growth and metastases.

No lentiviruses were used. This work was also performed using RM1-CDUPRT/GFP cells implanted in the prostate of C57BL/6 mice, which were then treated intratumorally with AdmIL12, AdmIL18 or both, with or without the administration of 5FC.
The results are shown in the poster (AACR, 2007, Khatri et al) (Appendix II). We have shown that the combination of GDEPT and cytokine therapy was the most effective, both against the local prostate cancer growth and against lung colony formation, indicating an improvement in the distant bystander effect when immunotherapy is given concurrently with GDEPT.

We have also shown that the combination therapy led to a marked immune infiltration into the prostate tumors including both CD4 and CD8 positive T cells, F4/80 positive macrophages and asialo GM positive NK cells (Figure 1, Appendix I). In addition, serum analysis for cytokine profile was performed in mice after treatment (day 17) and clearly showed a skewing towards TH1 type immune responses (see poster). This may have explained the increased efficacy of the combination therapy. In addition, the treatment was repeated in mice in order to examine its effects on mouse survival. Survival benefit from combined GDEPT and immunotherapy exceeded that from either GDEPT or immunotherapy alone (see poster).

Studies in TRAMP mice were also attempted, but the mice developed the tumors sporadically, and much later than anticipated. Hence we have not been able to repeat these experiments within the time frame of the grant.

TASK 4. **Tissue slice work**: Assess the ability of lentivirus encoding green fluorescence protein (GFP) under a prostate directed promoter from Dr Paul Rennie, Vancouver to express GFP in human tissue slices. (Months 24-33).

Although we acquired a Krumdeick Tissue slicer (Birmingham, Alabama) through an equipment grant (Rebecca L Cooper foundation) to conduct these experiments, and Dr Khatri attended training in Dr David Curiel's laboratory (Birmingham, Alabama), we have not been able to perform these experiments due to lack of time.

Task 5. Collate data, prepare reports and manuscripts (Months 33-36)
Some papers have been written (please see Appendix II)

KEY RESEARCH ACCOMPLISHMENTS:

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP and RM1-GFP/LacZ, RM1-GFP/mIL12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.
- Shown that CDUPRT-GDEPT + 5FC is associated with a ***local bystander effect***
- Shown that CDUPRT-GDEPT + 5FC given into the prostate is also associated with a ***distant bystander effect***, resulting in a reduction of the growth of pseudometastases of RM1 cells in the lungs of C57BL/6 mice, and with immune cell infiltration in the prostate
- Shown that CDUPRT-GDEPT +5FC effect is associated with infiltration of the tumors with immune cells, especially, macrophages, NK cells and CD4+ cells. Also the treated tumors show greater levels of necrosis and apoptosis, disrupted vasculature and enhanced proliferation in comparison to control tumors. *This is encouraging as it suggests further augmentation of the immune responses when CDUPRT-GDEPT is used in conjunction with the cytokine gene therapy.*
- Established that RM1-GFP/mIL12 and RM1-GFP/mIL18 secrete biologically active mIL12 and mIL18 respectively by *in vitro* assay.
- Shown that RM1-GFP/mIL12 and RM1-GFP/mIL18 inhibit tumor take rate and tumor growth after implantation either subcutaneously or in the prostate of C57BL/6 mice.
- Shown that a combination of intratumoral therapy of RM1-GFP/CDUPRT prostate tumors using pVITRO2.mIL12 + pVITRO2.mIL18 inhibits pseudometastases of RM1 cells in the lungs of C57BL/6 mice.
- Shown that a combination of intratumoral therapy of RM1-GFP/CDUPRT prostate tumors using pVITRO2.mIL12 + pVITRO2.mIL18 in mice treated with 5FC inhibits prostate tumor growth more than GDEPT alone.
- Shown that a combination of intratumoral therapy of RM1-GFP/CDUPRT prostate tumors using AdmIL12 + AdmIL18 in mice treated with 5FC inhibits prostate tumor growth, locally and within the lungs, more than either treatment alone, and has greater survival benefit than either alone.
- We have demonstrated that the treatment effects are reflected in increased immune infiltration and a skewing of the cytokine profile in serum towards TH1 immune responses.

REPORTABLE OUTCOMES:

- Khatri A, Zhang B, Doherty E, Chapman J, Ow K, Pwint H, Martiniello-Wilks R, Russell PJ. Combination of cytosine deaminase with uracil phosphoribosyl transferase leads to local and distant bystander effects against prostate-cancer in C57BL/6 mice. *J Gene Medicine*, 2006;8(9):1086-96.
- Russell PJ, Khatri A. Novel gene-directed enzyme prodrug therapies against prostate cancer. *Expert Opin on Investigational Drugs.* Invited review, 2006; Aug;15(8):947-61. Review.
- Khatri A, Russell PJ (2007), Targeted Gene directed enzyme prodrug therapies to tackle diversity and aggression of late stage prostate cancer, *Discovery Medicine* 2007;7(37):39-45.
- Russell PJ, Khatri A, Zhang B, Doherty E, Ow K, Chapman J, Martiniello-Wilks R. Gene directed enzyme directed prodrug therapy using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase leads to a distant bystander effect in mouse models of prostate cancer. Proceedings of the 96th American Association for Cancer Research, Anneheim, April, 2005
- Khatri A, Russell PJ , Zhang B, Doherty D, Ow K, Chapman J, Martiniello-Wilks R. Combination of cytosine deaminase with uracil phosphoribosyl transferase leads to local and distant bystander effects against RM1 prostate cancer in C57BL/6 mice. Fourth Annual Australasian Gene Therapy Society Meeting, Melbourne, April, 2005. Journal of Gene Medicine 2005;7(8):1129–1130.
- Jeet V, Ow K, Doherty E, Curley B, Russell PJ, Khatri A. Broadening of transgenic adenocarcinoma of the mouse prostate (TRAMP) model to represent late stage hormone refractory cancer. Proceedings of the 98th American Association for Cancer Research, LA, April, 2007.
- Khatri A, Husaini Y, Ow K, Chapman J, Russell PJ**.** Combined gene therapy with cytosine deaminase plus uracil phosphoribosyl transferase and immunostimulatory IL12 and IL18 cytokines for treating prostate cancer in C57BL/6 mice. Proceedings of the 98th American Association for Cancer Research, LA, April, 2007.
- Khatri A, Husaini Y, Russell PJ. Murine CTLL-2 cells respond to mIL12: Prospects for developing an alternative bioassay for measurement of murine cytokines IL12 and IL18. MS Number: JIM-D-07-00048 Submitted to J Immunol Methods, 2007.
- Jeet V, Ow K, Doherty E, Curley B, Russell PJ, Khatri A. Broadening of transgenic adenocarcinoma of the mouse prostate (TRAMP) model to represent late stage androgen independent cancer. For submission to Prostate.
- Khatri A, Husaini Y, Chapman J, Ow K, Russell PJ. Increased bystander effects of gene therapy with cytosine deaminase plus uracil phosphoribosyl transferase by concomitant treatment with virally delivered immunostimulatory IL12 and IL18 cytokines for treating prostate cancer in C57BL/6 mice. Paper in preparation.
- Russell PJ, Husaini Y, Chapman J, Ow K, Perryman L. Combined gene therapy with cytosine deaminase plus uracil phosphoribosyl transferase and immunostimulatory IL12 and IL18 cytokines for treating prostate cancer in C47BL/6 mice. Innovative Minds in Prostate Cancer Today (IMPaCT) submission-Atlanta, USA 2007 Sept 07, USA.

CONCLUSIONS:

- At this stage of the work, we have 3 papers published, 1 paper submitted, 2 papers in preparation and four abstracts.
- We have proof of principle that CDUPRT-GDEPT + 5FC is an effective therapy against RM1 tumor cells grown in the prostate of C57BL/6 mice. This treatment is associated with a local bystander effect, and stimulates a distant bystander effect as evidenced by inhibition of pseudo-metastasis formation in the lungs after intravenous injection of RM1 cells.
- We have clearly shown using CTLL2 cells that mIL12 and mIL18 together exhibit increased effects over either alone in increasing cellular proliferation.
- We have shown that RM1-GFP/mIL12 and RM1-GFP/mIL18 cell lines express biologically active cytokines that inhibit tumor take and tumor growth after implantation under the skin or in the prostate.
- We have shown that mIL12 and mIL18 synergize in preventing pseudo-metastases from RM1 cells in the lungs.
- This synergy is improved when mIL12 and mIL18 are delivered by adenoviruses.
- We have shown that trimodal therapy, GDEPT (CDUPRT/5FC) with mIL12 and mIL18, given into the prostate, result in an increased effect against local tumor growth and against metastases.

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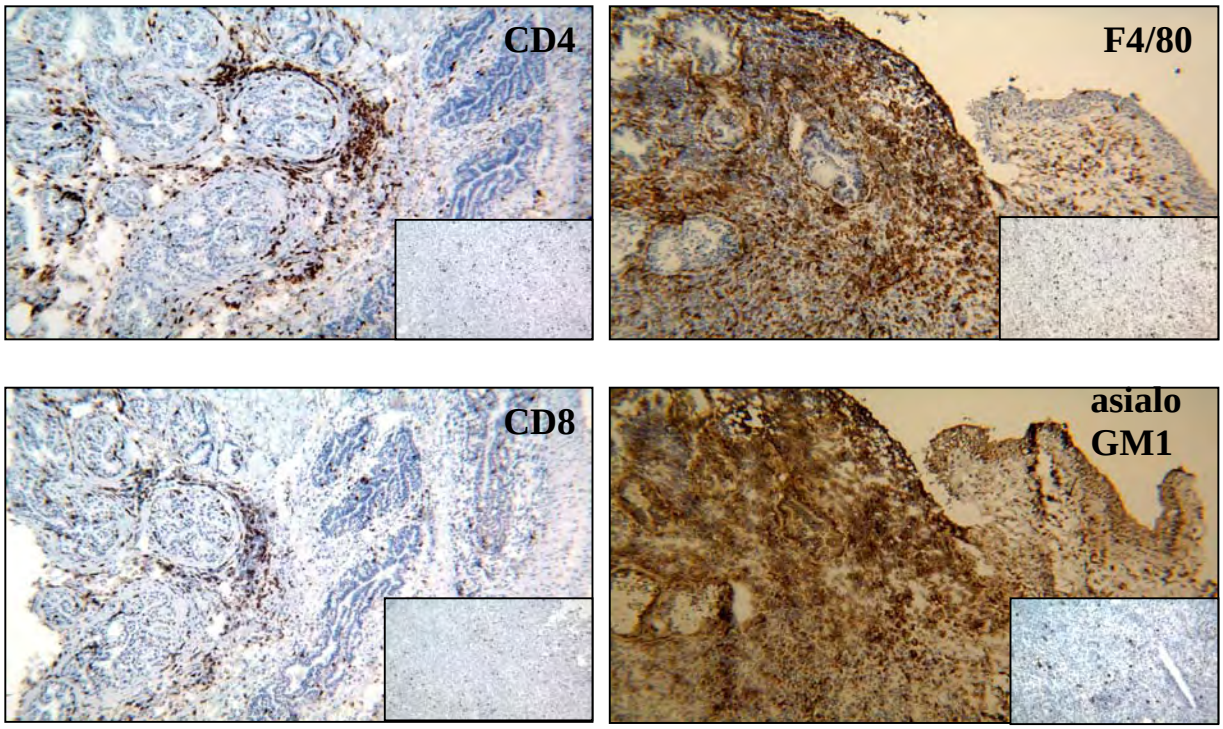
Voeks, D, **Martiniello-WilksR** and **Russell PJ**. Derivation of MPR and TRAMP models of prostate cancer and Prostate cancer metastasis for evaluation of therapeutic strategies. *Urologic Oncology*, 2002b;**7**:111-118.

APPENDICES:

Appendix I contains figures
Appendix II Publications and papers in preparation

APPENDIX I

Figure 1. Immunohistochemical staining for the presence of immune cell infiltrates in the prostates of mice treated with a combination of CDUPRT-GDEPT (with 5FC) and AdmIL12 and ADmIL18 as described in poster (Appendix II, AACR, 2007). Staining for T cells (CD4, CD8), macrophages (F4/80) and NK cells (Asialo GM1) is shown. Inserts show staining in sections from prostate tumors sections from mice treated with control virus (Ad GFP) without GDEPT.



APPENDIX II Reportable Outcomes

- Khatri et al. *J Gene Medicine*, 2006;8(9):1086-96.
- Russell PJ, Khatri A. *Expert Opin on Investigational Drugs.* Invited review, 2006; Aug;15(8):947-61. Review.
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Editorial Manager for Molecular Therapy
Manuscript Draft

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Article Type: Regular Article

Section/Category:

Keywords: GDEPT; CDUPRT gene; RM1 model; 5 fluorocytosine; local bystander effect

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Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against RM1 prostate-cancer in mice.

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Running Title: CDUPRT-GDEPT using the mouse RM1 prostate cancer model

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Abbreviations Used: AR, Androgen refractory; AS, Androgen sensitive; PC, Cancer of the prostate; FBS, Fetal bovine serum; GDEPT, Gene directed enzyme prodrug therapy; HSVtk, Thymidine kinase gene from Herpes Simplex Virus; PNP, purine nucleoside phosphorylase. HRPC, Hormone refractory prostate cancer.CD, cytosine deaminase; UPRT, uracil phosphoribosyl transferase; CDUPRT, cytosine deaminase in combination with uracil phosphoribosyl transferase. 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; GFP, Green Fluorescent protein; HPLC, High performance liquid chromatography; 5FdUMP, 5-fluoro-2'-deoxyuridine 5'monophosphate; 5FUTP, 5-fluorouridine 5'-triphosphate; 5FUMP, 5-fluorouridinemono-phosphate; ICGJ, Intercellular gap junctions; NK cells, Natural killer cells.

ABSTRACT
We aimed to evaluate the efficacy of gene–directed enzyme prodrug therapy (GDEPT) using cytosine deaminase in combination with uracil phosphoribosyl transferase (CDUPRT) against intra-prostatic mouse androgen-refractory prostate (RM1) tumors in immunocompetent mice. The product of the fusion gene, CDUPRT, converts the prodrug, 5-fluorocytosine (5FC) to 5-fluorouracil (5FU) and other cytotoxic metabolites that kill both CDUPRT-expressing and surrounding cells, via a 'bystander effect'. RM1 cells were stably transfected with plasmids containing green fluorescence protein (GFP)/CDUPRT, GFP or GFP/LacZ genes. CDUPRT expression in RM1-GFP/CDUPRT cells or tumors was confirmed by enzymic conversion of 5FC to 5FU, using HPLC. Treatment of mice bearing intra-prostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. A 'local bystander effect' was seen, even though only 20% of the cells expressed CDUPRT. More importantly a significant reduction in pseudo-metastases of RM1 cells in lungs indicated a 'distant bystander effect'. Immunohistochemical evaluation of the treated tumours showed increased necrosis and apoptosis, with decreased tumor vascularity. There was also a significant increase in tumour-infiltration by macrophages, $CD4^+$ T and natural killer cells. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate-tumors and lung pseudo-metastases via immune mechanisms involving necrosis and apoptosis.

KEYWORDS: GDEPT; CDUPRT gene; RM1 model; 5 fluorocytosine; local bystander effect

INTRODUCTION

Of several GDEPT systems under investigation for PC (1), we are evaluating a fusion gene constructed by combining cytosine deaminase (CD) with uracil phosphoribosyltransferase (UPRT). Cytosine Deaminase is an enzyme of bacterial or fungal origin that converts the non-toxic pro-drug, 5 fluorocytosine (5FC) to 5-fluorouracil (5FU). 5FU is further modified by cellular enzymes to pyrimidine antimetabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) that inhibit DNA and RNA synthesis. Thus, 5FU should be effective in killing both dividing and non-dividing cells, an important factor, given the low percentage (2%) of dividing cells in PC (2). Despite the success of 5FU-chemotherapy for gastrointestinal, head and neck malignancies, it has shown a poor therapeutic index for other cancers primarily because some tumor cells develop immunity to 5FU toxicity. A major factor responsible is the low efficiency of conversion of 5FU into its toxic metabolites; the rate-limiting step is the formation of an intermediary metabolite, 5-fluorouridinemono-phosphate (5FUMP) involving a series of enzyme-catalyzed reactions (3). This is circumvented by the ability of UPRT to convert 5FU directly to 5FUMP leading to more efficient production of toxic metabolites (4) and hence increased sensitization (up to 15 fold) of the UPRT-transduced cells to 5FU (5). Use of UPRT in conjunction with CD sensitizes cancer cells to low doses of 5FU and 5FC (3,6,7) and results in greater anti-tumor efficacy compared with CD-GDEPT alone (7,8). Only limited studies have investigated the effects of CDUPRT GDEPT *in vivo* against PC.
Given that a 100% gene transfer efficiency with currently used gene delivery technology is unattainable *in vivo*, an important advantage of GDEPT is derived from the local bystander effect engendered; comprehensive cell killing is achieved without the need to express the gene in all cells (9,10). The local bystander effects associated with 5FC/5FU-based GDEPT is due to the ability of 5FU to freely diffuse between cells (7,11). However, the metabolites of 5FU can only

move through gap junctions (12) and therefore the extent of the bystander effects relies on the incidence of gap junctions in the cells. While the combination CDUPRT-GDEPT clearly results in a more efficient conversion of 5FU to its toxic metabolites, it may also lead to depletion of the cytoplasmic pool of freely diffusible 5FU that could compromise the bystander effect. Clearly, this needs to be investigated individually for different cell types. Although *in vitro* studies have shown that the local bystander effect associated with CDUPRT-GDEPT varies depending upon the cancer cell properties (7,11,13), none have evaluated the bystander effects against PC *in vivo*. Hence, in this study we sought to assess the local bystander effects of CDUPRT *in vitro* and *in vivo* against murine PC. Some GDEPTs including CD- and UPRT- lead to tumor cell killing in the remote locations, culminating in a *distant bystander effect* (1,14,15), which is mediated via treatment specific anti-tumor immune responses. To date there are no reports of any preclinical studies evaluating the distant bystander effects generated using the fusion gene, CDUPRT, so, we assessed its ability to produce a similar '*distant bystander effect'.*

An immunocompetent mouse model of syngeneic PC was used to examine the ability of locally given GDEPT to exert local and distant bystander effects. An androgen-refractory PC cell line (RM1) (16) was implanted into the prostate or tail vein of C57BL/6 mice leading to tumor formation and experimental lung metastasis, respectively, in an aggressive and reproducible manner (17).

RESULTS

1. Efficacy of CDUPRT-GDEPT *in vivo*

Optimization of 5FC dose: An important feature of CDUPRT GDEPT is that both 5FC and 5FU (FDA approved drugs) can be used as prodrugs for this system. We chose 5FC as it is efficiently metabolized by the fusion gene and is non-toxic at the therapeutic doses used. We first determined the maximum non-toxic dose of prodrug usable in our model. C57BL/6 mice could tolerate 5FC at doses up to 500 mg/kg/mouse, given intraperitoneally (ip) every day for 14 days without systemic toxicity as shown by examination of Hematoxylin and Eosin (H&E) stained major organs (Fig.1A) and by serum analysis for biochemical markers of kidney and liver function (Urea Creatinine, ALP, ALT and AST (Fig.1B). Neither serum markers, nor histology differed between 5FC-injected versus control mice, suggesting that there was no detectable toxicity from 5FC even at the highest dose tested. There was no loss of weight or condition in the treated mice.

Generation of stably transformed RM1 cell lines expressing GFP, GFP CDUPRT or GFPLacZ: RM1 cells were stably transformed to express GFPCDUPRT, GFP or GFPLacZ (RM1GFP, -CDUPRT or -LacZ) and gene expression of the transgenes was established. For CDUPRT*, in vitro* RM1-CDUPRT cultures and tumors harvested after subcutaneous (sc) and intraprostatic (Iprost) growth in C57BL/6 mice were shown by HPLC to have the capacity to convert 5FC to 5FU, confirming CDUPRT expression and importantly, there was no loss of activity *in vivo* over the duration of the experiments (data not shown). GFP expression in all cell lines was confirmed by UV microscopy and flow cytometry.

Evaluation of RM1 cells for assessment of CDUPRT GDEPT: Given that some cells are resistant to 5FU therapy, toxicity of 5FU to RM1 cells was assessed *in vitro* to evaluate the suitability of our model for evaluating *CDUPRT GDEPT.* Cultured RM1 cells were subjected to different doses of 5FU for 1 week (data not shown) and then assessed for viability by cell counting via trypan blue exclusion. There was almost complete eradication of cells at dose > 10μg/mL (77μM, 85% cell death) at 72 h post treatment. Cytotoxic effects were seen (~57% cell death compared with untreated controls) even at 1μg/mL (7.7μM) by day 7, which was lower than that for the human PC DU145 cells (17μM) (13) but higher than the acceptable therapeutic range of 5FU in humans (1.9 ± 0.3μM). Although, doses lower than 7.7μM were not tested, these data suggested that RM1 cells are moderately sensitive to 5FU toxicity. When tested for 5FC sensitivity, control

RM1-LacZ cells were resistant up to the highest dose tested (100μg/ml) but RM1-CDUPRT cells were susceptible to cell killing even at 3μg/ml showing ~34 fold enhancement of 5FC sensitisation of RM1–CDUPRT cells (data not shown). This clearly established the suitability of our model for assessing CDUPRT-GDEPT.

GDEPT using RM1CDUPRT/5FC: To establish that RM1-CDUPRT cells could effect GDEPT *in vivo* in the presence of 5FC, mice were implanted Iprost with RM1CDUPRT or RM1-LacZ tumors and injected ip with 5FC or saline. GDEPT in the RM1-CDUPRT/5FC group was very effective with almost complete absence of growth in the prostate compared to mice in control groups (RM1-LacZ/5FC, RM1-CDUPRT/saline) (Fig.2A)(P=0.009). Histology of RM1-CDUPRT tumors from mice treated with saline (6 mice) or 5FC (10 mice) showed a highly vascularized and viable tumor (Fig 2B) in the former, whereas treatment with 5FC resulted in necrosis (Fig 2C), with loss of prostate tissue architecture (Fig 2C, inset). All **t**umors from CDUPRT/saline mice showed >80% viability, with <10% tumor necrosis or haemorrhagic necrosis (necrosis due to disruption of vasculature). In contrast, mice given 5FC had no tumors (in 6 cases), or <10% viable tumor (4 cases) with >30% necrosis, and >60% haemorrhagic necrosis in the latter (Table 1). Other tissues examined histologically (kidney, lung, spleen, liver), showed no abnormalities (data not shown), indicating the tumor-specific nature of the cytotoxic effects of the treatment with no apparent systemic toxicity. Finally, HPLC analysis of sera from mice from RM1-CDUPRT/5FC and RM1-LacZ/5FC groups showed no detectable levels of 5FC and 5FU in either (data not shown).

2. Local bystander effect on RM1 growth

Once the efficacy of the CDUPRT GDEPT was established, any local bystander effects resulting from the treatment were assessed *in vitro* and *in vivo*.

When examined *in vitro,* medium collected from RM1-CDUPRT/5FC but not from RM1-LacZ/5FC cells prevented the growth of RM1 cells ($p<0.0001$) indicating a CDUPRT-specific bystander effect (Fig 3A). This was assessed *in vivo* by implanting mixtures of RM1-CDUPRT and RM1-GFP cells Iprost in different proportions, followed by treatment with 5FC. Prostate tumor volumes measured at necroscopy indicated that the minimal proportion of RM1-CDUPRT cells required to produce a therapeutic effect was 20% ($p=0.01$) (Figure 3B).

Apoptosis after GDEPT: To investigate how GDEPT mediates cell death *in vivo*, the extent of apoptosis was evaluated by TUNEL assay (34) on tumors from different treatment groups (Fig 4A, Table2). Scattered apoptotic cells were seen throughout the tumors of RM1-LacZ/5FC or RM1-CDUPRT/saline controls. Apoptosis was markedly increased in tumors of the GDEPT groups in a dose dependant manner. An increase of 1.8 fold was seen in 100% RM1-CDUPRT/5FC group compared with 100%RM1-GFP/5FC group ($p=0.0001$), in both necrotic and non-necrotic areas.

Tumor vasculature after GDEPT: We noted in the previous experiment that CDUPRT-GDEPT tumors were characterised by extensive haemorrhagic necrosis suggesting that the treatment may have disrupted the tumor vasculature. To investigate this possibility, vascular analysis (anti-CD31 staining) of the entire tumor section was performed. Any stained endothelial cells or clusters separated from adjacent microvessels were included and counted as one microvessel, whereas infrequent CD31-positive macrophages and plasma cells were excluded from the analysis. Neither vessel lumens nor red blood cells were used to define a microvessel (18). CDUPRT/FC caused a reduction in the vascularity of the tumors by more than 3X compared to the control RM1-GFP tumors (Table 2, Fig. 4B, $p=0.006$). This extent of reduction increased with increasing number of CDUPRT expressing cells (Table 2) suggesting the vasculature disruption to be CDUPRT-GDEPT-specific and possibly involved in the enhancement of its cytotoxicity.

3. Distant bystander effects of CDUPRT-GDEPT

Next, we investigated if CDUPRT GDEPT leads to a similar '*distant bystander effect'* by assessment of whether killing of cells in the prostate by CDUPRT-GDEPT would have any effect on the growth of pseudometastases of the parental RM1 cells in the lung. Three independent experiments were performed and data from a representative experiment is shown. The number of lung colonies in mice in the RM1-CDUPRT/5FC group was much lower than those in the control RM1-GFP/5FC or RM1-CDUPRT/saline groups (Fig.4A and 4B). The lungs of the control groups were completely covered with RM1 tumors in all mice and this posed logistical problems. Therefore, they were arbitrarily given the value of 450 colonies (Fig. 4B) on the basis of the average of counts done in 3 representative mice from each control group. While similar trends were seen in all three experiments, there were variations in lung colony numbers between experiments. In one experiment, 50% of the GDEPT treated mice had no lung colonies compared with no such mice in the control groups (data not shown). This suggests that a '*distant bystander effect'* may have prevented the growth of RM1 lung colonies.

Immunohistochemical studies of RM1 tumors The distant bystander effect is characterized by infiltration by the immune cells e.g. macrophages, $CD8^+$ $CD4^+$ T, B and NK cells in tumors undergoing GDEPT (19-22). Immunoperoxidase staining was used to assess infiltration by macrophages, $CD8^+$ $CD4^+$ T, and NK cells in Iprost tumors from different treatment groups (Table 2). Increasing numbers of $CD4^+$ T cells were detected in all three RM1-CDPURT/5FC treatment groups (B, C and D) compared with RM1-GFP+5FC control group (A) ($p<0.05$). Further, this recruitment was enhanced by 7X in tumors from 100% RM1-CDPURT/5FC group compared with 2X in 20% and 10% mixed cell tumors (Fig 6A). In contrast, staining for $CD8a^+$ cells was minimal in all groups. Although not statistically significant, increasing numbers of infiltrating $F4/80^+$ (macrophages) and Asialo-$GM1^+$ (including NK) cells were detected in tumors of the three RM1-CDPURT+5FC groups (B, C) compared with the control group (A, Table 2); representative sections from treated tumors are shown in Fig. 6. This CDUPRT-specific dose dependant increase in $CD4^+$, macrophages and NK cells is a strong indication of the involvement of the immune system in local and distant bystander effects.

DISCUSSION

We previously reported that a single dose of PNP-GDEPT was effective against orthotopic RM1 tumors, leading to increased survival (17,23). We have now explored a new GDEPT system, CDUPRT fusion gene with the prodrug, 5FC. We wanted to examine the local and distant bystander effects of CDUPRT-GDEPT in the context of an intact immune system particularly as it was implicated in developing systemic anti-tumorigenicity with CD/5FC and UPRT/5FU GDEPTs (24,25). We used a syngeneic immunocompetent mouse model of orthotopic and pseudo-metastatic murine PC (26) which was further characterized by us (27). This model is susceptible to other GDEPTs (HSV/TK, PNP) (17,23,28). Further, RM1 cells are mildly immunogenic and express MHC class 1 molecules making them susceptible to immune system mediated cell killing (28,29). Further when tested for sensitivity to 5FU, sensitivity of RM1 cells (57% cell killing at 1μg/mL (7.7μM) was comparable to that of mouse mammary carcinoma (1μM) and mouse lymphoma cells (10μM) (30). This was higher than the accepted therapeutic range in humans and offered an ideal therapeutic window for assessment of CDUPRT GDEPT. When tested for 5FC sensitivity, the control RM1LacZ cells were not affected even at 100μg/mL of 5FC but efficient cell killing was seen for RM1-CDUPRT cells at low doses of >3μg/mL, well within the accepted steady state levels in humans (50μg/ml) (31). The 5FC related systemic toxicities were undetectable in mice with no loss of condition at the experimental dose (500 mg/kg/mouse) commonly used in other studies (6).

The *in vivo* efficacy of the CDUPRT GDEPT against RM1 Iprost tumors was unequivocally proven and supports other studies showing the efficacy of the combination against different types of cancers including PC (6,7,13). Treatment was associated with a local bystander effect (Fig 3), and stimulated a distant bystander effect as evidenced by inhibition of pseudo-metastases in lungs (Fig 5). *This is the first in vivo demonstration of local and distant bystander effects in PC using the fusion gene, CDUPRT.* Bystander effects of CDUPRT GDEPT are operated via two independent mechanisms: diffusion of cytotoxic 5FU and transfer of the cytotoxic fluoronucleotides (FUMP and FUTP) via intercellular gap junctions (ICGJ) to surrounding cells. This provides for the cell type-based variation in the bystander effects. Indeed, *in vitro* local bystander effects caused by CDUPRT-GDEPT are described for glioma (6), glioblastoma (11) and colon carcinoma (7), generally when 2- 10% of cells expressed the transgene. This variability was due to differences in 5FU sensitivity (11) and ICGJ status of the cells (6). Miyagi *et al* (13) showed that ICGJ lacking DU145 human PC cells showed only 30% cell killing when 10% cells were expressing CDUPRT, *in vitro*. While CDUPRT- GDEPT was more effective than CD-GDEPT against DU145 xenografts, the local bystander effects for these two GDEPTs against DU145 cells showed an opposite trend *in vitro*. The authors postulated that although the active phosphorylated metabolites of the CDUPRT GDEPT could not diffuse efficiently in DU145 cells which lack ICGJ (12), a more efficient conversion of 5FC into 5FU sensitized the cells to 5FU leading to enhanced anti-tumor killing *in vivo*.

In our study, we demonstrated the presence of the local bystander effect *in vitro* qualitatively. Use of cell mixtures containing different proportions of RM1 cells with RM1-GFP-CDUPRT cells did not yield significant data as the RM1 cells grow twice as fast compared with RM1CDUPRT cells both *in vitro* and *in vivo* (data not shown). Interestingly, when investigated *in vivo,* the data clearly showed a bystander effect when 20% of the cells expressed the transgene. This was significant given that the faster growth rate of RM1 cells would bias the results against the demonstration of the bystander effects. Other factors that could affect the extent of bystander effect in our system: A. Moderate sensitivity of the RM1 cells to 5FU toxicity (7.7 μM compared with 17μM in 5FU resistant DU145 cells) B. Diminution of the pool of available 5FU due to efficient conversion to its metabolites (4) C. RM1 cells lead to aggressive non-differentiated tumors *in vivo* (27) and it is known that loss of ICGJ is a critical step in progression to human prostate neoplasia (32). It is likely that intercellular gap junctions are absent or present at low concentration in RM1 tumors and hence the bystander effects are not as dramatic as expected from other studies (6,7).

Our laboratory is the first to describe the distant bystander effect of CDUPRT-GDEPT in vivo. We have shown that operating the CDUPRT-GDEPT in the prostate of mice results in a considerable suppression of parental RM1 colony formation in the lungs.

Two factors may contribute to this. The first is due to chemically induced responses related to RM1-sensitivity to systemically given 5FC or 5FU introduced into the bloodstream during GDEPT. It was unlikely that 5FC contributed to toxicity as RM1 cells were resistant to 5FC even at 100μg/mL and serum and histological analysis of organs from mice given 5FC did not show any 5FC related toxicity; also 5FC or 5FU could not be detected in the blood sera of the treated mice. This suggested an alternate mechanism such as anti-tumor immune responses triggered during GDEPT may have mediated cell killing of RM1 cells. It was postulated (33) that a distant bystander effect is induced due to release of cytokines via immune cell activation, triggered by GDEPT induced tumor destruction. This leads to hemorrhagic necrosis that then allows more immune cells to infiltrate the tumor. A number of studies support this theory (reviewed in (1). Our studies show that immune cells infiltrating the primary tumor included $CD4^+$T cells, macrophages and NK cells, suggesting their involvement in anti-tumor activity in this system. This is a wider repertoire of immune cells compared to when either GDEPT is used alone. The distant bystander effects from CD- and UPRT-GDEPTs are mediated by the immune system

(14,15,34). Thus UPRT expressing murine colon carcinoma cells in syngeneic immunocompetent mice led to tumor regression when treated with 5FU compared with wild type tumor cells (14). Treated mice rejected the wild type- but not irrelevant syngeneic tumor cells. This distant bystander effect was less efficient in nude mice suggesting that αβ T cells were involved. In comparison, in a rat model that mimics liver metastases of colon carcinoma, CD GDEPT led to regression of CD positive tumors (25,34) and resistance in treated rats to wild type challenge. Immunodepletion studies showed that NK cells were involved. This would suggest that NK and αβ T cells are implicated in CD and UPRT GDEPT effects, respectively. Hence, when the two systems are combined the immune system may be augmented by the inclusion of a larger repertoire of anti-tumor immune cells. Our preliminary analyses support this, but immunodepletion studies are needed to ascertain the mechanisms involved.

Hemorrhagic necrosis was a remarkable feature of CDUPRT/5FC treated RM1-GFP/CDUPRT tumors (Fig 2C,Table 1) suggesting that endothelial cells in the tumor vasculature were susceptible to GDEPT. This disruption could be mediated by the cytokines released due to the stimulation of the immune system (33) or due to the toxicity of 5FU released in the tumor microenvironment (35). G1-arrest in endothelial cells was observed when treated with 5FU (39). In our study, haemorrhagic necrosis increased in a dose dependant manner with GDEPT treatment (Table 2), strongly suggesting that disruption of tumor vasculature may have contributed further to the tumor regression in CDUPRT treated mice. The elucidation of the mechanism of this disruption however, was beyond the scope of this study.

Killing of tumor cells by anticancer therapies such as chemo-, radiation-, immuno- or suicide gene therapy is predominantly mediated by triggering apoptosis. Our data indicated that apoptosis was involved in the death of RM1 tumor cells expressing CDUPRT (Fig. 5) and again the dose dependence of this effect suggested that it was CDUPRT GDEPT-specific. This accords with other studies showing the involvement of apoptosis in cell killing mediated by various GDEPTs such as HSV/TK, PNP and CD (23,36-38).

Although the CDUPRT-GDEPT has not yet been trialled in humans, the safety of the CD-GDEPT has been well reported in PC patients (39). In this preclinical study, there was no apparent toxicity to other organs indicating that CDUPRT-GDEPT has excellent safety features against normal host tissues. There is increasing emphasis on use of gene therapy in concert with other strategies, including strategies that enhance anti-tumor immunity. Our data indicate that CDUPRT/5FC GDEPT significantly suppressed the growth of RM1 cells producing both a local and a distant bystander effect by mechanisms of killing that involve necrosis and apoptosis possibly mediated by the immune responses. This proof of principle study will form the basis of the future studies assessing the impact of combination of CDUPRT GDEPT with immunotherapy.

1. MATERIALS AND METHODS

Cell lines and mice

The RM1 (16) and the transformed derivatives (RM1-CDUPRT, RM1-GFP and RM1-LacZ) were cultured in Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen, CA, USA) containing 10% fetal calf serum (Invitrogen, CA, USA) with hygromycin (hygro) (Invitrogen, CA, USA) at a concentration of 800μg/ml for the later. Male C57BL/6 (6-8wk) mice were bought from Labortory Animal services, Perth, AU.

Transfection of RM1 cells

RM1 cells were transfected with pVITRO2-GFP/CDUPRT, pVITRO2-GFP/LacZ (Invivogen, CA, USA) or pVITRO2-GFP (InvivoGen, CA, USA) to generate stable transfectants (RM1-CDUPRT, RM1-LacZ and RM1-GFP). The pVITRO2-GFP/CDUPRT was constructed by

excision of CDUPRT (CodA::upp) gene from pORF-codA::upp (InvivoGen, CA, USA) using *NcoI* and *NheI* restriction enzymes followed by its ligation into complementary sites in the pVITRO2-GFP/LacZ (InvivoGen, CA, USA). The construct was authenticated by restriction enzyme digestion using *NcoI* and *NheI*. For transfections, cells were transfected with complexes formed by combining 15µl Lipofectamine 2000 (Invitrogen, CA, USA) and 5µg plasmid DNA according to manufacturer's instructions. Stable clones were maintained under hygro B selection (800µg/mL). GFP expression was used to sort the cells (FACscan sorter, BD, USA) for high levels of GFP expression and to eliminate drug-resistant, non-expressing clones.

Optimization 5FC dose *in vivo*

Mice injected ip with 150, 300 and 500 mg/kg/mouse/day of 5FC (InvivoGen, CA, USA) or saline for 13 days were monitored daily for general behaviour and condition and their body weights were measured every second day. Toxic effects were monitored by assessment of liver and renal function via evaluation of the levels of urea, creatinine, alkaline phosphatase (ALP), alanine amino-transferase (ALT) and aspartate amino-transferase (AST) in the serum samples by the South East Area Laboratory Services (Prince of Wales Hospital, AU) using standard techniques.

Assessment of CDUPRT expression *in vitro* and *in vivo*

To assess the functionality of CDUPRT in RM1-CDUPRT cells/tumours, an HPLC based assay was developed measuring the catabolism of the prodrug 5FC to 5FU. Homogenates of RM1-CDUPRT or –LacZ cells sc/intraprostatic (Iprost) RM1-CDUPRT or –LacZ tumors (Liquid N_2) were generated and lysis was completed by 3 cycles of freeze thawing. Cell debris was removed by centrifugation (15,000g, 10 min) followed by determination of the protein content of the supernatants (BCA protein estimation kit, Pierce, IL, USA). 100µL of the supernatant was then incubated with 900 µL of 0.5 mM 5FC at 37^{o}C. At 24 h, the samples were stored at –20^{o}C, after a 10 min incubation at 85^{o}C. Reversed phase liquid chromatography employing a C18 column under isocratic conditions (0.05% Trifluoroacetic acid in H_2O) at a flow rate of 0.7 mL/min was used to analyse samples (10 µL). Absorbance was measured at 275 nm. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC and 5FU.

GDEPT *in vivo*

Iprost injections were performed with $5x10^3$ RM1-CDUPRT (test) or RM1-LacZ (control) cells in the subcapsular region of the prostate surgically after opening the abdomen in C57BL/6 mice as previously described (23). Day 4 onwards, 5FC or saline was administered ip at 500 mg/kg/day for 14 days. Mouse weights were recorded twice/week. At necroscopy (day 18), the prostate tumor volumes were determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$ where d_1 and d_2 are diameters at right angles (40). The tumor and other organs (kidney, lungs, liver, heart, spleen) were fresh frozen or paraffin-embedded for subsequent histological and immunohistochemical studies. Mouse serum was stored at –80^{o}C until analysis.

Local bystander effect on RM1 growth

In vitro: Conditioned media from RM1-CDUPRT cells +/- 5FC (at 1mM) or RM1-LacZ cells+5FC were harvested 48 h after the addition of 5FC. These mixed with an equal proportion of fresh medium were then incubated with RM1 parental cells ($5x10^3$ cells/well in a 96 well plate). Cell viability was determined at 72 h using the WST1 proliferation assay (Roche, Sydney, AU) according to the supplier's instructions.

In vivo: RM1-CDUPRT cells and RM1-GFP cells were mixed in different proportions and implanted Iprost in mice followed by 5FC treatment daily for 14 days. As data from our earlier experiments showed the GDEPT to be specific to RM1-CDUPRT/5FC group, to minimize the mouse usage, the saline controls included earlier were not performed. On day 19, tumors and other organs were processed as described above.

Effects of RM1-CDUPRT plus 5FC on growth of pseudometastases in the lungs

Mice were injected Iprost with $5x10^3$ RM1-CDUPRT or RM1-GFP cells. On day 4, mice received $2.5x10^5$ RM1 cells iv and 5FC treatment daily ip for 15 days. On day 19, their lungs were fixed in Bouin's fixative and the RM1 colonies were counted as previously described (23).

Immunohistochemical analysis of orthotopically implanted prostate tumors

For detection of Immune infiltration: Snap frozen tissues were embedded in Optimal Cutting Temperature compound (Tissue Tek), sectioned (5 μm) and acetone fixed. To block endogenous peroxidase/biotin and non-specific monoclonal antibody binding, sections were incubated sequentially with 1.5% H_2O_2 (5 mins), avidin block (10 mins), biotin block (10 mins), 3% bovine serum albumin in PBS (5 mins). Sections were then stained using rat α mouse -CD4 (BD-PharMingen, 1:100); -CD8a (BD-PharMingen, 1:200); -F4/80 (BD-PharMingen,1:800), -CD31 1:300, BD-Pharmingen) and rabbit α mouse AsialoGM1 (Dako,1:400) as relevant by incubating for 45 minutes at room temperature followed by incubation with secondary antibodies, α -rat (1:200) and α-rabbit (1:200) and the ABC complex for 15 minutes. The standard ABC detection system, the diaminobenzidine (DAB) as the chromagen and Harris hematoxylin as counterstain were used.

For detection of Apoptosis: Tissues fixed in 10% neutral buffered formalin (Amber Scientific) were paraffin-embedded (Tissue Tek-VIP (Sakura). Dewaxed paraffin sections (Histochoice, Ambresco) were rehydrated through graded series of ethanol and finally in PBS before blocking. Apoptotic cells were detected using the Tunel assay kit (Roche) as described (41) after antigen retrieval.

Scoring of positive stained cells in the immunostained sections was performed by light microscopy. After initial scanning under x100 magnification, positive stained cells in ten fields under x400 (0.15 mm^2) magnification were counted and the mean number/high power field (HPF ± SEM) was determined. Because of the high number of positive apoptotic cells, a higher magnification (x620) was used.

Data analysis

A one-way analysis of variance (ANOVA) was performed (GraphPad PRISM V4) if the data in multiple groups were normally distributed. A Turkey's post-test was performed if the ANOVA indicated a significant difference ($p<0.05$) between treatments.

ACKNOWLEDGEMENTS

This work was supported by DOD Prostate Cancer grant, DAMD-17-1-02-0107, and by the DOD fellowship, DAMD-17- 01-1-0083.

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Table 1: Effects of CDUPRT-GDEPT on prostate histology.

Treatment 5 x 10^3 RM1-GFP/CDUPRT (Iprost)	Viability	Necrosis	Haemorrhagic[a] Necrosis
+ saline ip 15 days (n=6)	>80%	<10%	<10%
+ 5FC (500 mg/kg/day) ip daily for 15 days (n=4)	<10%	>30%	>60%
+ 5FC (500 mg/kg/day) ip daily for 15 days (n=6)	No	No growth	No growth

[a] Haemorrhagic necrosis was defined by necrotic cell death observed in areas with vascular damage, primarily observed in tumours treated with CDUPRT-GDEPT.

Table 2. Immunohistochemical analysis of infiltrating immune cells, endothelial cells (vascularity) and apoptotic tumor cells in RM1-GFP/CDURPT+5FC and control tumors.

Treatment	Number of Infiltrating immune cells								Vasculature (endothelial cells)		Apoptotic tumor cells	
	$CD4^+$		$CD8a^+$		$F4/80^+$		$AsialoGM1^+$		CD31		Tunel assay	
	Mean[a]	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
(A) RM1-GFP+5FC (Control)	4.55	0.39	1.26	0.40	10.85	1.90	13.05	1.10	18.03	2.89	47.03	3.74
(B) 10%RM1-GFP/CDURPT mixed with 90%RM1-GFP +5FC	9.43	3.36	4.25	1.27	20.53	0.76	14.93	1.82	13.82	2.09	65.30	0.62
(C) 20%RM1-GFP/CDURPT mixed with 80% RM1-GFP +5FC	9.40	3.46	1.73	0.90	16.23	2.38	16.20	2.76	8.33	0.13	72.97	2.17
(D) RM1-GFP/CDURPT (100%) +5FC	32.9	8.9	1.70	1.70	22.00	4.71	20.27	3.94	5.67	0.35	84.25	0.75
One way ANOVA P value	0.003		0.19		0.002		0.04		0.0061		0.0001	

[a] Mean number of positively stained cells/high power field (HPF $\pm$ SEM) (x400) (0.15 mm square) from a count of 10 fields. SEM: Standard error of the mean.

Figure Legends

Figure 1: Analysis of toxicity of 5FC in C57BLl/6 mice. Mice (4 mice /group) were injected ip with 100, 150 and 500 mg/kg/day of 5-FC or saline for 13 days. At necroscopy, heart, liver, lung and spleen were harvested and analysed by H&E staining for toxic effects of the drug. (A)The four panels show different organs from mice treated at the highest dose, 500mg/kg/day. Insets in each panel represent the corresponding organ from control mice treated with saline. There was no detectable toxicity even at 500mg/kg/day. (B) serum analysis for biochemical markers of kidney and liver function (ALP: Alkaline Phosphatase, ALT: Alanine Amino Transferase and AST: Aspartate Amino Transferase is shown. The reference represents the values for a normal mouse.

Figure 2: *In vivo* evaluation of therapeutic effects of CDUPRT-GDEPT using RM1-GFP/CDUPRT cells +5FC

(A) 5x10^3 cells were implanted orthotopically in the prostate of C57BL/6 mice. 4 days post-implantation the prodrug 5FC or saline were administered ip at 500mg/kg/mouse/day for 13 days. At necroscopy (day 17), prostate tumor volume was measured. Prostate volumes were determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles.

(B) H&E staining of paraffin-embedded orthotopic RM1-CDUPRT prostate tumor sections show that treatment with saline resulted in highly vascularized viable tumor (x40), (C) Treatment with 5FC resulted in extensive necrosis (black arrow) and haemorrhagic necrosis (red arrow) (C x40), with some loss of prostate tissue architecture (insert, x10).

Figure 3: Evaluation of "local bystander effect" of CDUPRT *in vitro* and *in vivo.*

(A) *In vitro*: RM1-GFP/CDUPRT cells were grown in presence or absence of 5FC for 48 h, then the supernatants were collected. Supernatants (conditioned media, CM) from RM1-GFP/LacZ cells grown in the presence of 5FC, served as controls. Parental RM1 cells were then treated with these CMs at 50% concentration. The bystander effect was demonstrated by cell killing of parental RM1 cells using theCM from RM1CDUPRT cells using WST1 viability assay (see methods).

(B) *In vivo*: Local bystander cell killing effects of the CDUPRT suicide gene is demonstrated. RM1-GFP/CDUPRT cells were mixed with RM1-GFP cells in different proportions and 5x10^3 total cells were implanted in the prostate of C57BL/6 mice. The mice were injected intraperitoneally with the prodrug, 5-fluorocytosine (5FC) at 500 mg/kg/day, from day 4 onwards

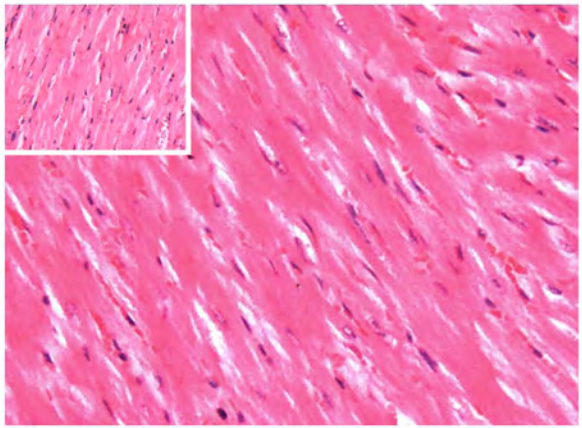
daily for 15 days. Mice were sacrificed on day 19, and their prostate volumes determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles.

Figure 4: Assessment of apoptosis and vascular integrity in test (CDUPRT + 5FC) or control (RM1-GFP + 5FC) tumors (all x40): (A) Tunel positive cells in test tumor vs Inset, Tunel stain control tumor; (B) Decrease in CD31 positive cells in RM1-GFP/CDUPRT + 5FC tumor vs Inset, CD31 positive cells in control tumor.

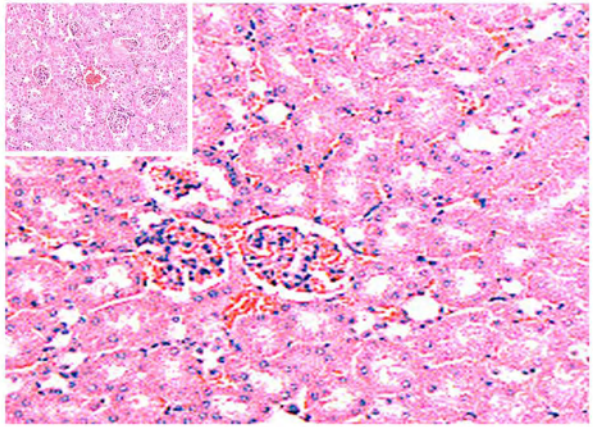
Figure 5: Distant bystander effect of CDUPRT suicide gene *in vivo*. RM1-GFP/CDUPRT cells ($5x10^3$) were implanted in the prostate of C57BL/6 mice. Four days later, the mice were injected intravenously with the parental RM1 cells at the dose of $2.5x10^5$ cells/mouse. The mice were injected with the prodrug 5FC from day 4 onwards daily for 15 days. At necroscopy, the lungs were harvested, stored in Bouin's reagent and colony counts were performed. (A) Scatter graph showing the number of lung colonies in different treatment groups (B) Photographs of lungs demonstrating the distant bystander effect of CDUPRT GDEPT on pseudometastases.

Figure 6: Evaluation of immune cell infiltration after treatment in test (CDUPRT + 5FC) or control (RM1-GFP + 5FC) tumors (all x40) (A) Cluster of CD4 positive T cells (brown colour) in test tumor; Inset few CD4 positive T cells in control tumor.; (B) Cluster of F4/80 positive macrophages in test tumor. vs Inset: control tumor; (C) Asialo-GM1 positive immune cells clustered at the tumor periphery in test tumor vs Inset, Control tumor.

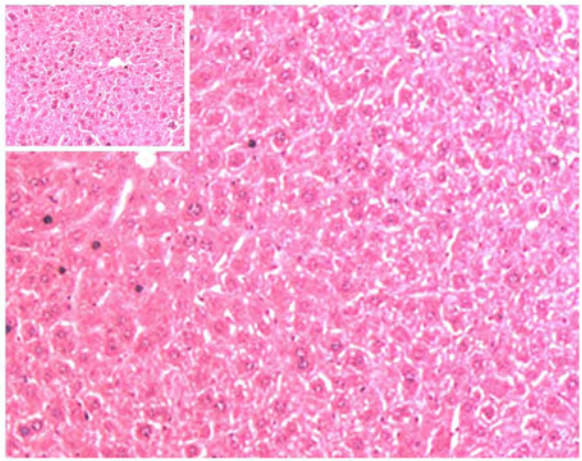
A



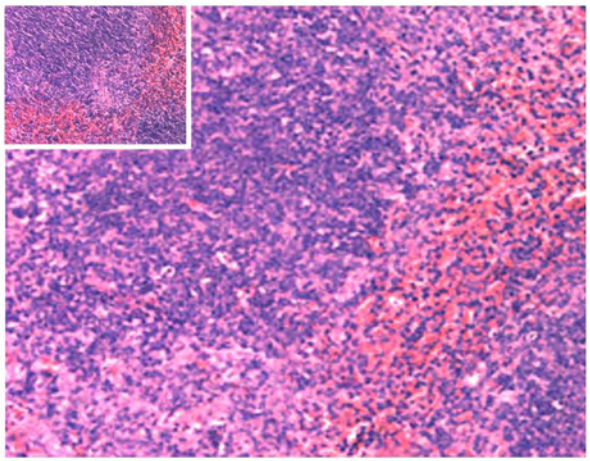
Heart



Kidney



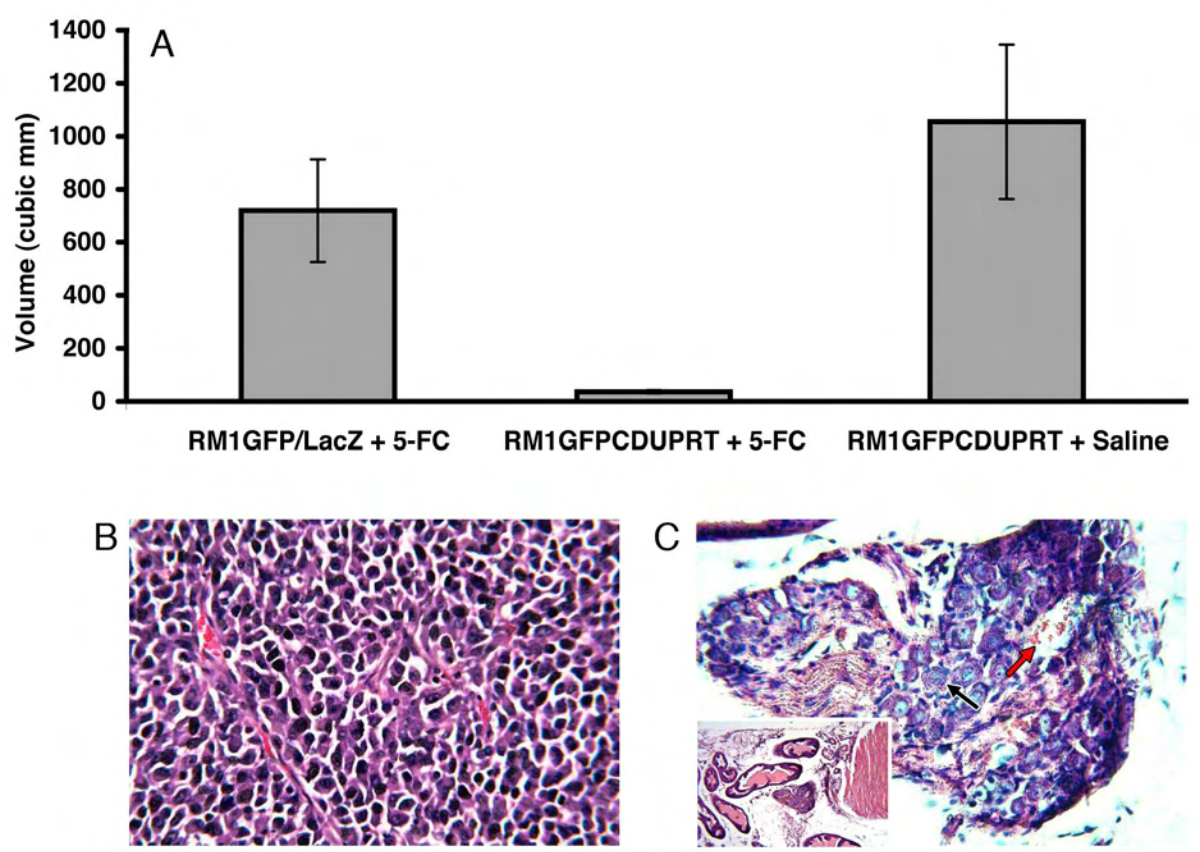
Liver

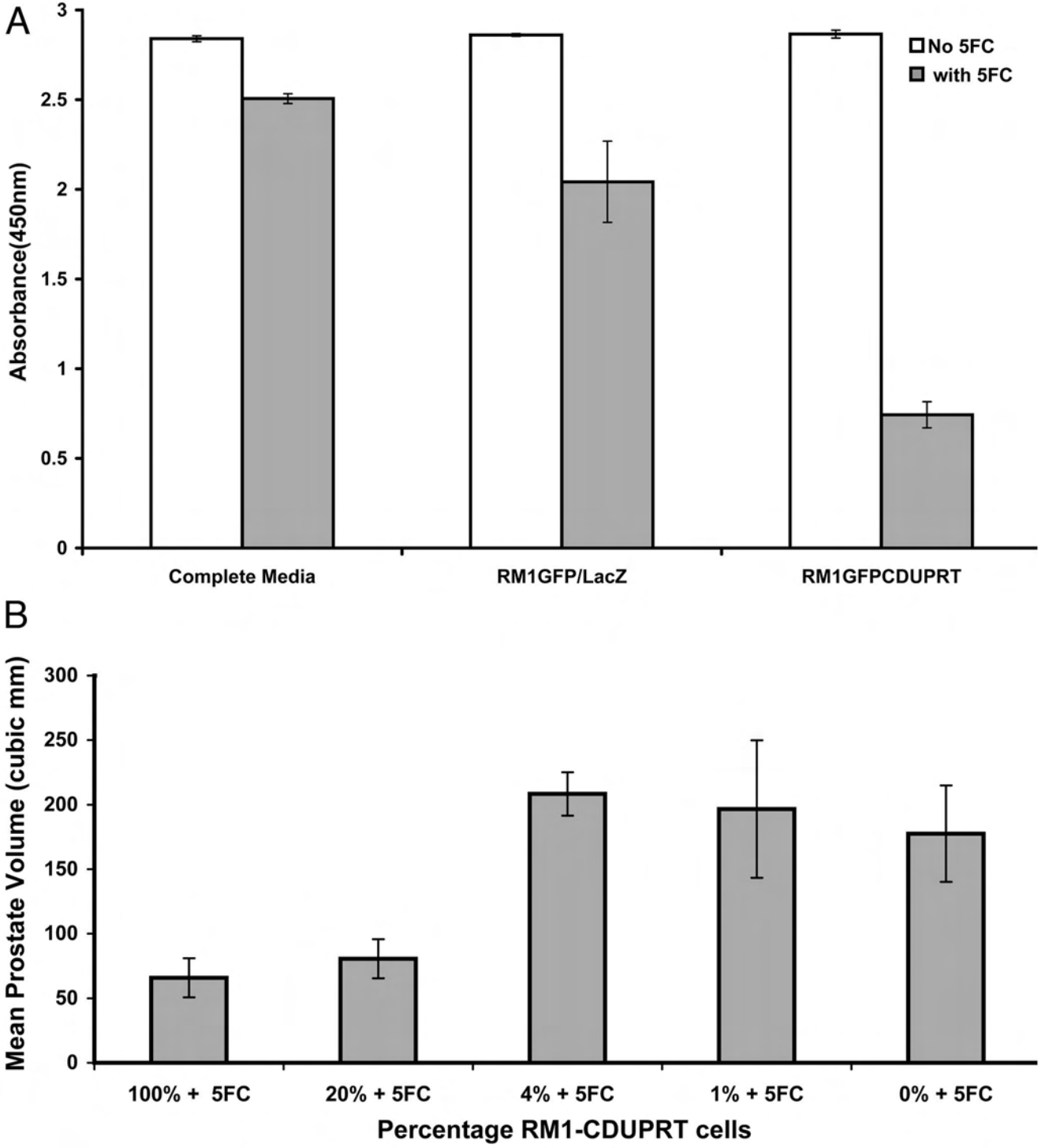


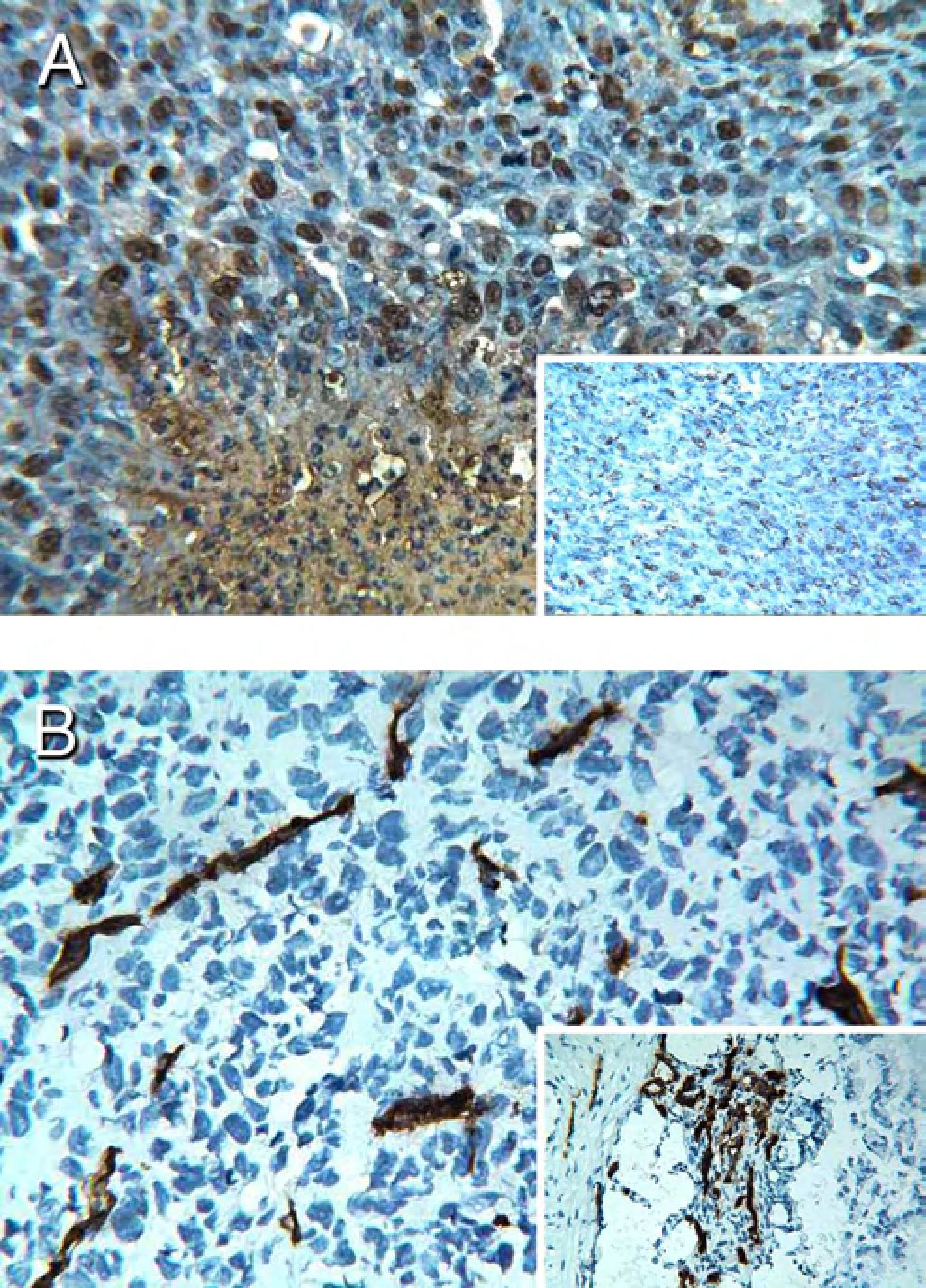
Spleen

B

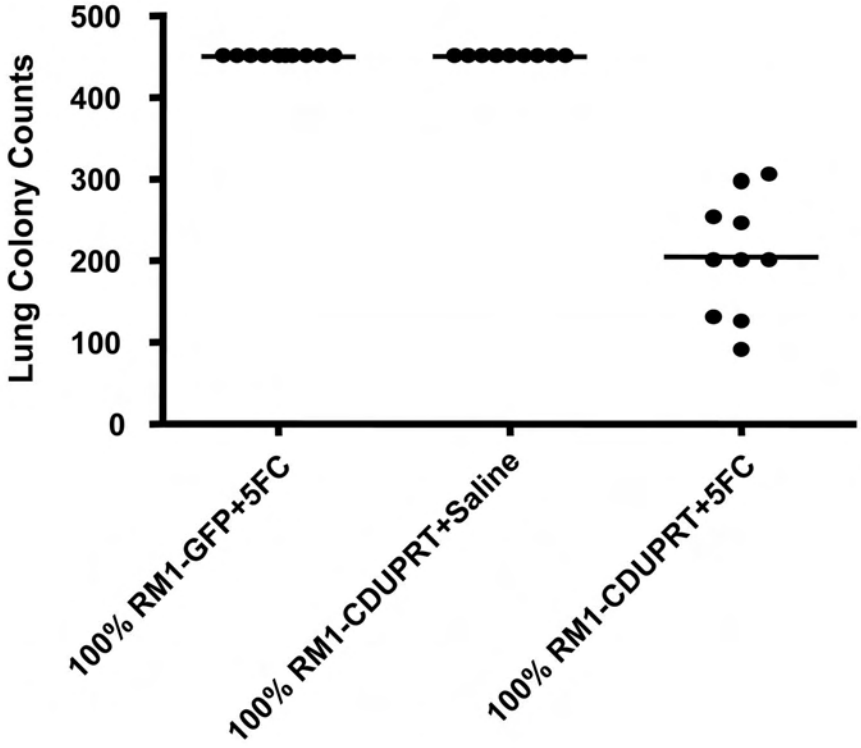
Treatment	Urea (mmol/L)	Creatinine (umol/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Reference	**1.4-5.5**	**18-80**	**35-96**	**17-77**	**54-298**
Control	6.7 ± 0.3	29.3 ± 3.3	54 ± 1.7	32.5 ± 5.7	155 ± 25
500 mg/kg/d	6.8 ± 0.7	25.3 ± 1.8	55.7 ± 4.9	19 ± 1.5	57.3 ± 11.9


A
1400
1200
1000
800
600
400
200
0
Volume (cubic mm)
RM1GFP/LacZ + 5-FC
RM1GFPCDUPRT + 5-FC
RM1GFPCDUPRT + Saline
B
C

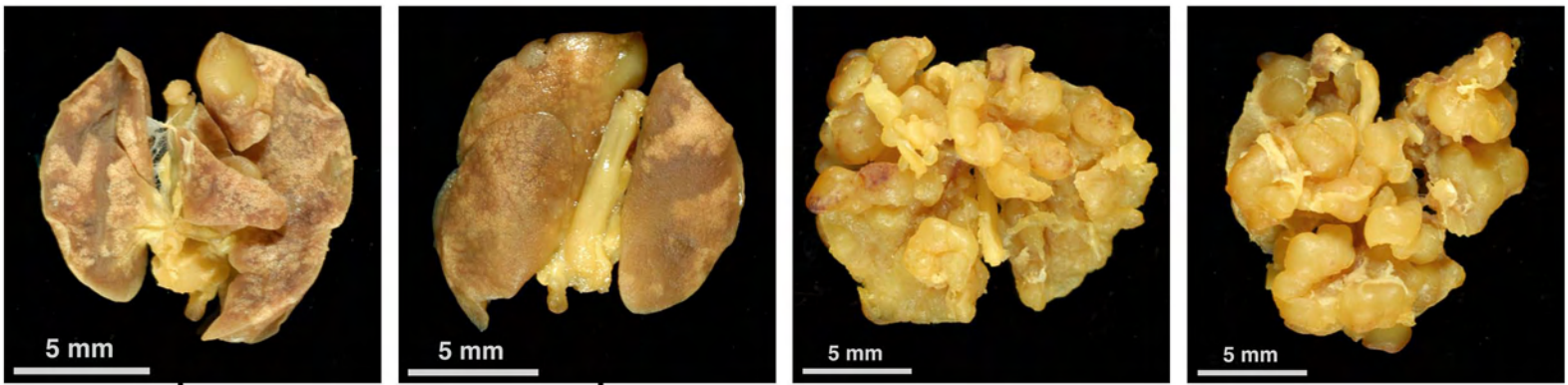

A
Absorbance(450nm)
3
2.5
2
1.5
1
0.5
0
No 5FC
with 5FC
Complete Media
RM1GFP/LacZ
RM1GFPCDUPRT
B
Mean Prostate Volume (cubic mm)
300
250
200
150
100
50
0
100% + 5FC
20% + 5FC
4% + 5FC
1% + 5FC
0% + 5FC
Percentage RM1-CDUPRT cells

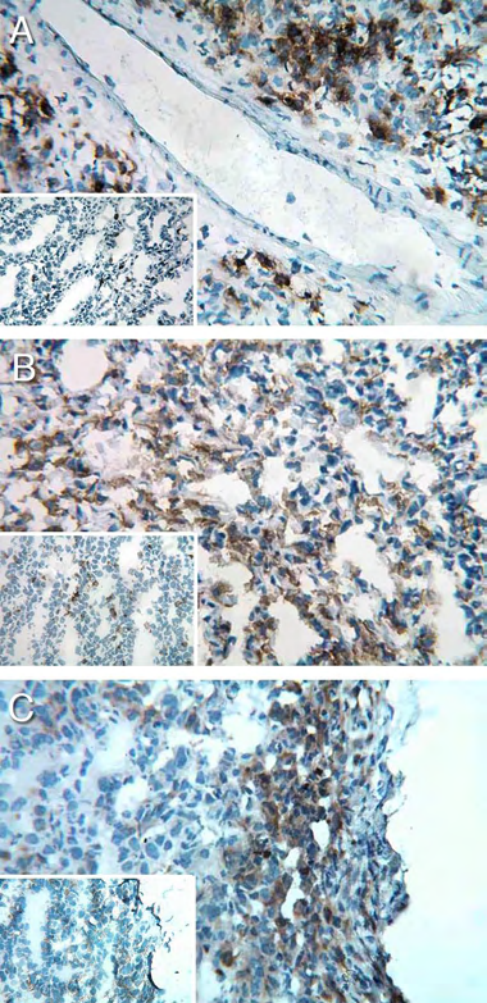

A
B

A


Lung Colony Counts
500
400
300
200
100
0
100% RM1-GFP+5FC
100% RM1-CDUPRT+Saline
100% RM1-CDUPRT+5FC
Treatment Group

B


5 mm
5 mm
5 mm
5 mm
RM1CDUPRT/5FC
RM1CDUPRT/saline
RM1GFP/5FC


A
B
C

Expert Opinion

informa
healthcare

Oncologic

Novel gene-directed enzyme prodrug therapies against prostate cancer

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There is no effective cure for late-stage hormone (androgen) refractory prostate cancer. Although chemotherapy offers palliation to these late-stage patients, it also leads to systemic toxicities leading to poor quality of life. Clearly, the focus is on the development and evaluation of novel biologically relevant alternatives such as cytoreductive gene-directed enzyme prodrug therapy (GDEPT). With the current limitations of effective gene delivery *in vivo*, the *in situ* amplification of cytotoxicity due to bystander effects of GDEPT has special attraction for patients with prostate cancer, the prostate being dispensable. This review focuses on the development, application and potential of various GDEPTs for treating prostate cancer. The current status of research related to the issues of enhancement of *in situ* GDEPT delivery and prostate cancer-specific targeting of vectors (especially viral vectors) is assessed. Finally, the scope and progress of synergies between GDEPT and other treatment modalities, both traditional and alternate, are discussed.

Keywords: 5-fluorocytosine, cytochrome 450, cytosine deaminase, fludarabine phosophate, ganciclovir, GDEPT, gene-directed enzyme prodrug therapy, nitroreductase, prostate cancer, purine nucleoside phosphorylase, thymidine kinase

Expert Opin. Investig. Drugs (2006) ***15****(8):xxx-xxx*

1. Introduction

Prostate cancer (PC) is currently the second most common cause of cancer death in men in Western society. Despite continuous efforts towards improving diagnosis and prevention [1,2], > 30,000 men die of PC annually in the USA [3]. Based on the fact that the prostate will not grow, develop or function without androgenic hormones and an intact androgen receptor, the standard initial systemic therapy for locally advanced and metastatic PC is androgen ablation therapy [4]. The median response period lasts ≤ 30 months but almost inevitably, the cancer recurs having lost its androgen dependence (hormone-refractory PC [HRPC]) [4]. Patients with HRPC show diverse presentations, elevated prostate-specific antigen (PSA) with or without metastases, radiological progression (soft tissue and bone) and clinical progression represented by worsening pain and urinary obstruction. HRPC is highly resistant with chemotherapy yielding a median survival of ≤ 12 months. As a result, its primary use has been to palliate symptoms secondary to this disease [5-7]; however, recently, the use of the microtubule inhibitor docetaxel in HRPC patients resulted in a mean increase in lifespan of ~ 2 months in ~ 40% of cases [8,9]. New strategies are needed, either for use alone or in combination with other therapies. There are several gene therapy approaches for PC [10-12] including cytoreductive approaches such as suicide gene therapy (e.g., gene-directed enzyme prodrug therapy [GDEPT]), induction of apoptosis [13,14], oncolytic virotherapy [15], immunomodulatory approaches [16] and those that induce modification of

signalling pathways [17]. Clinical trials using adenoviral vectors for PC have recently been reviewed [18] and current protocols are available [19]. This review focuses on one approach, and presents an overview of current and novel GDEPTs. The fact that the prostate is a nonessential organ offers an added advantage for such applications to the treatment of PC.

2. GDEPT

The lack of selectivity of toxins for treating cancer has led to new strategies that allow direct delivery of the drug to the cancerous tissue, avoiding side effects to normal tissues. Earlier strategies used monoclonal antibodies to target chemotherapeutic agents to cancers [20] and, recently, GDEPTs have evolved. The concept is to deliver an enzyme (e.g., bacterial, yeast or viral) that is absent or expressed at a low concentration (human) in normal tissue directly into the cancer that can then convert a systemically administered nontoxic prodrug into a cytotoxic substance. In theory, the 'drug' is delivered directly into the cancer, killing the cancer cells locally as well as decreasing systemic effects. The major advantage of GDEPT-based cancer therapy is the *in situ* amplification of the cytotoxic effects due to the local and distant bystander effects engendered in most systems.

3. GDEPT delivery

Despite *in situ* amplification of the cytotoxic effects of GDEPT, delivering enough enzyme into the cancer is the biggest hurdle to effective GDEPT in the clinic. Many viral and nonviral gene delivery systems have been used. Viral vectors rely on the biological ability of the viruses to enter cells efficiently; these include integrating viruses (such as retro- [21], lenti- [22,23] and adeno-associated viruses [12]) and non-integrating Vaccinia- [24] and Herpes [25], non-replicative human adeno- (Ads) [18], ovine atadeno- (OAdV) [26,27], conditionally replicative adeno- (CRAds) [18] or other oncolytic viruses [15]. Nonviral delivery systems rely on the binding of recombinant DNA to positively charged lipid bilayers, cationic polymers and peptides that can enter cells by endocytosis or by fusion with the cell membrane. They include the use of naked DNA [28], liposomes [29,30], cationic amphiphiles [31], proteins and, recently, nanoparticles [32]. A recent report described the best performing polymer, C32, from a library generated to deliver DNA to cancer cells that was shown to be 26-fold more effective than naked DNA after intratumour injection with no toxicity [33]; this was successfully used to deliver DNA encoding the A chain of diphtheria toxin to LNCaP PC xenografts, 40% of which regressed [33]. Although more safe than the viral vectors, nonviral transfer systems lead to short-term expression and are relatively inefficient in transfecting different cell types, both *in vitro* and *in vivo* [34,35]. In comparison, viral vectors are more efficient for gene delivery *in vitro* and *in vivo*. The properties of viruses that make them suitable for use as delivery vehicles are reviewed elsewhere [36-38]. In fact, > 60% of gene therapy trials (GDEPT: 7.4%) have employed viral vectors. This review focuses on the viral vector-mediated GDEPT delivery. The choice of delivery system is also dictated by the characteristics of the tumour. The percentage of dividing cells is low in PC [39], thus both the delivery system and the activated prodrug need to target both nondividing and cycling cells to be effective for its treatment. Hence, adeno- and related viruses (e.g., OAdV) that can infect dividing and nondividing cells are a preferred method for gene delivery to PC [40]. The transient nature of the Ad-mediated episomal gene delivery is suitable for treating cancer; the persistence of gene expression is not required as the ultimate aim is to achieve killing of the transduced cells. In contrast, diseases such as congenital genetic diseases require persistent gene expression (integrating vectors; e.g., adeno- and retroviral vectors) to achieve a long-term effect. Recently, cancer-specific replication competent oncolytic viruses (e.g., Conditionally replicating Ads, CRAds) have been used in the clinic with promising results to further enhance the delivery of the transgene to the tissue [41,42].

4. Local and distant bystander effects

The main attraction of GDEPT is the local bystander effect (not all cancer cells need to express the transgene to be killed by the drug generated) [43,44]. In general, the toxic product/s of the administered prodrug can enter the surrounding non-transduced cells passively or via gap junctions, causing their death (**Figure 1**), effectly leading to *in situ* amplification of cytotoxicity. The characteristics of the prodrug–drug system used is an important consideration in evaluating the extent of the bystander effect. Killing tumour cells using GDEPT also induces systemic antitumour responses including host immune responses that can decrease metastatic growth of cancer cells at distant sites (**Figure 1**), which is known as the distant bystander effect. Depending on the GDEPT system used, this involves NK cells and macrophages as well as T cells [45,46]; it has implications for treating metastatic cancer.

5. Targeting GDEPT to PC

To date, viral vectors have mostly been given directly into the prostate of PC patients as it is not an essential organ and is accessible, thus avoiding issues associated with systemic delivery including hepatic clearance [40]. For disseminated disease, systemic delivery would be preferable. Treatments targeted (preferably) to cancer cells or to the target tissue should deliver DNA to tumour cells with high efficiency, induce minimal toxicity and avoid gene expression in healthy tissues. Cancer gene therapy based on tissue-restricted expression of a cytotoxic gene should achieve a superior therapeutic index over unrestricted methods.

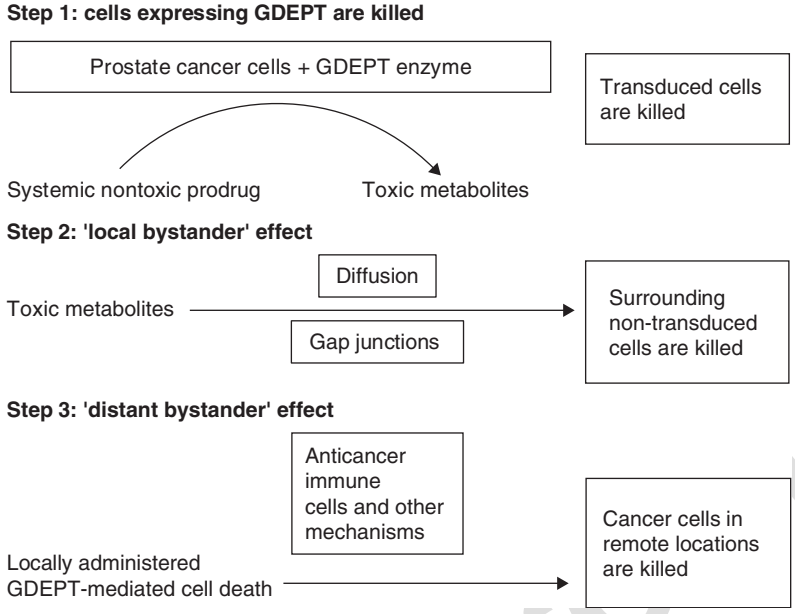

Figure 1. General mechanism of GDEPT mediated local and distant bystander effects, showing three of the steps involved in cell killing mediated via GDEPT.
GDEPT: Gene-directed enzyme prodrug therapy.

5.1 Transcriptional targeting

Prostate targeting of viral vectors can be achieved transcriptionally by using a promoter or enhancer that is active in prostate cells only to regulate the transgene expression [47]. Early work used the PSA promoter [47] and increased specificity was found when an additional enhancer from PSA (PSE) was also used [48]; however, recent imaging studies reveal that transgenes under the PSA promoter are also expressed in the lungs of nude mice [49]. Similarly, promoters or enhancers [50] from the prostate-specific membrane antigen (*PSMA*) gene effectively target PC [26,51]. Novel chimaeric enhancers composed of two modified regulatory elements controlling the expression of PSA and PSMA give high prostate tissue specificity when tested *in vitro* and in the prostate of mice [52]. Many promoters target androgen-dependent PC (probasin PB) [53,54], PSA and PSMA [50,55]; combinations that work in HRPC are rarer. The authors have used a promoter–enhancer combination that is effective in androgen-dependent and HRPC xenografts containing a partial promoter from PB together with an enhancer from either SV40 virus [27] or (more effectively) from *PSMA* gene [26]. Other groups have identified and characterised a prostate-specific androgen-independent protein-binding site in the PB promoter [56] or have used a combination of promoter/enhancer of *PSMA* gene together with the Cre-lox system [57]. The promoter/enhancer region from prostate-secretory protein (PSP94) has been successfully used to direct the formation of PC in transgenic mice and may have potential for targeting GDEPT [58]. Caveolin-1 is overexpressed in metastatic and HRPCs and in tumour-associated endothelial cells, and is the subject of study for directing GDEPT [59,60] to PC; human osteocalcin promoter (OC) has been used to target bony metastases of PC [61,62]. Combinations of promoters have been used to target PC (PSA) and bone stromal cells simultaneously with greater antitumour effects than when targeting only one cell type [61].

5.2 Transductional targeting

Alternatively, viral vectors can be targeted by changing their tropism [63-65,66]. For the most commonly used Ad vectors, genetic retargeting strategies include fibre or fibre knob chimaerism, genetic incorporation of targeting ligands in the fibre or other capsid locales or a combination of both. Alternatively, bifunctional adapter molecules, which bind to the adenoviral particle on one side and to the targeted receptor on the other, have been employed. Strategies are used to block the adenovirus interaction with its cognate receptor (cognate receptor for adenovirus [CAR]), which is highly expressed on liver cells, to avoid the hepatic tropism associated with the use of human adenoviruses. In this regard, other non-CAR-specific viruses, such as OAdV, have an advantage as they do not enter the cell via CAR receptors and are not liver tropic [67].

6. Enzymes and prodrugs used in GDEPT for PC

Enzymes for GDEPT and their requirements for use have been reviewed elsewhere [68]. They should catalyse specific reactions not catalysed by endogenous enzymes, and should be delivered in sufficient amounts to effect prodrug conversion. Those used for treating PC include non-mammalian enzymes, such as thymidine kinase from herpes simplex virus (HSV-tk), Varicella–zoster virus or the *Escherichia coli* enzymes, cytosine

deaminase (CD), uracil phosphoribosyl transferase (UPRT) or a fusion of the two genes (*CDUPRT*), purine nucleoside phosphorylase (PNP), nitroreductase (NTR) and the human enzyme, cytochrome P450 (CYP) [69-71]. The use of other systems, such as human thymidine phosphorylase [72] and deoxycytidine kinase (dCK) [69,73] and *Pseudomonas* carboxypeptidase G2/CMDA [74] or horseradish peroxidase/indole-3-acetic acid [75] is not widely reported for PC and will not be further discussed in this review.

The main requirements for prodrugs have been described elsewhere [68,69]. Briefly, they should be: able to cross the mammalian cell membrane for intracellular activation; a good substrate for the enzyme in question able to form a drug that is highly cytotoxic and metabolically stable; and be able to diffuse efficiently. There also needs to be a high differential between the prodrug and its corresponding drug. Importantly, the prodrug needs to be of low cytoxicity to normal tissues as it is given systemically. The site of enzyme expression (cytoplasmic/nuclear or mitochondrial) was found to be critical in the efficiency of the prodrug activation with NTR–CB1954–GDEPT, thus suggesting that the location of gene expression in the cell is an important variable that may require consideration with other GDEPTs [76]. Although GDEPT allows the concentration of the toxic drug in the tumour microenvironment, the ability of the prodrug and its toxic products to be retained in the cell long enough to achieve effective cell toxicity levels would further enhance its efficacy. Development and design of new drugs with greater retention within the cancer cells would be ideal for achieving effective killing of the tumours, a trait that would be of great benefit in treating solid tumours such as PC; however, it is also critical that the drug (once formed) can enter nearby cells to elicit a local bystander effect. This is one of the great advantages of the GDEPT system. The physicochemical properties that govern the pharmacokinetic characteristics of prodrugs and their effectors include overall lipophilicity, charge, rate of metabolism and ability to form reversible/irreversible complexes with cellular macromolecules [69]. The local bystander effect is strongly influenced by the size and metabolism of the drug formed. If it is a small molecule, such as 2-fluoroadenine (2FU), a toxic drug formed from the activity of PNP on fludarabine phosphate, it is likely to diffuse passively into nearby cells providing a strong local bystander effect, whereas drugs that are highly phosphorylated, such as triphosphates of ganciclovir (GCV) or its analogue acyclovir (ACV) from the activity of HSV-tk on these prodrugs, may require active transport or gap junctions (connexins) [77] to allow toxicity to occur in neighbouring cells. Such drugs may have a variable bystander effect depending on the targeted cancer cell type.

Given the complexity of designing a prodrug to fit the necessary criteria, most of those used to date have already been tested clinically as anticancer agents, where the pharmacokinetics and safety parameters are established. Generally, two classes of anticancer agents are used in GDEPT: antimetabolites, such as GCV and ACV; or alkylating agents (e.g., cyclophosphamide). The antimetabolites have a complex pathway of activation [68] and can induce resistance, whereas alkylating agents have the advantage that they can crosslink noncycling and cycling cells, thus targeting a larger population of tumour cells [68].

6.1 Thymide kinase and prodrugs

The most widely used GDEPT system is HSV-tk, which phosphorylates antiherpetic drugs (e.g., GCV or ACV) to the monophosphorylated form, which is subseuqently phosphorylated to the toxic triphosphate by mammalian enzymes. Incorporation of this triphosphate into guanine sites during DNA replication inhibits DNA synthesis. Resistance to HSV-tk/GCV can occur if the cancer cells are in G0 at the time of delivery [78]. ACV also inhibits DNA polymerase by preventing the elongation of the newly synthesised DNA, causing chain termination [79,80]. Improved therapy is achievable using mutant forms of HSV-tk that result in lower Michaelis rate constant (K_m) values for thymidine binding to GCV [81]. Moreover, many analogues of GCV have been prepared [69] that differ in lipophilicity from GCV and show different bystander effects. However, the intercellular transport of these depends on the presence of connexins in the tumour cells under treatment [79]. Several studies [83], including those using prostate-directed promoters such as that from PSA [84], describe the effective reduction of human PC xenografts through the use of the HSV-tk–GCV system. Adenoviral delivery of HSV-tk with GCV has been tested in Phase I clinical trials for PC and found to exhibit a satisfactory toxicity profile [40,60]. In most trials, GDEPT has been administered directly into the prostate; however, in a separate trial, HSV-tk under an OC promoter was injected directly into PC lymph nodes and bone metastases [85]. All of the patients tolerated the therapy; local cell death was seen in treated lesions in 7 (63.6%) patients and stabilisation of the treated lesion in 1 patient for 317 days. As expected, the levels of intrinsic OC expression and the *tk* gene transduction affected the *HSV-tk* gene expression in the clinical specimens. A further enhancement of viral delivery would be ideal to increase the benefit from such therapy.

Another application of a mutant form of HSV-tk has been as a reporter gene, which allows imaging of tumours treated by HSV-tk/GCV GDEPT by positron emission tomography [86]. This mutant has an increased ability to phosphorylate penciclovir, an analogue of GCV. Imaging, assessed using fluorodeoxyglucose and F-penciclovir, was successful in PC xenografts grown orthotopically. This methodology should provide a useful strategy for monitoring gene expression and therapeutic efficacy in future clinical protocols. Other prodrugs including 4-([2-chloroethyl](2-[^{11}C]ethyl)amino)-phenoxycarbonyl-L-glutaminc acid, a half mustard prodrug, have also been synthesised for use in imaging GDEPT by positron emission tomography [87].

Another source of *tk* gene is VZV. GCV and its analogues are poor substrates for this enzyme hence, the prodrug (6-methoxy purine arabinonucleoside is used. This is activated

by VZV-tk to ara-M-monophosphate) and then converted through a series of steps using mammalian enzymes to ara-ATP [68]. This system has been used to treat hepatocellular carcinoma [21] but its use for PC has not been described.

6.2 CD alone or with UPRT and 5-fluorocytosine

After HSV-tk, CD is the next most widely used enzyme. It catalyses the conversion of 5-fluorocytosine (5FC) to 5-fluorouracil (5FU), a FDA-approved therapeutic widely used for treating various cancers including PC [51,88]. CD–GDEPT has been safely trialed in Phase I studies either alone for colon carcinoma of the liver [89] or in combination with HSV-tk–GDEPT for local recurrent PC [41]. The mechanism is as follows (**Figure 2**): bacterial or yeast CD converts 5FC to 5FU (freely diffusible) whose metabolites (5-fluoro-2′-deoxyuridine 5′-monophosphate [5-FdUMP] and 5-fluorouridine 5′-triphosphate) damage both DNA and RNA, respectively. 5-FUTP can be incorporated into RNA in place of UTP, resulting in the inhibition of the nuclear processing of ribosomal and messenger RNAs, whereas 5-FdUMP irreversibly inhibits thymidylate synthase, preventing DNA synthesis (**Figure 2**). The rate-limiting step in the generation of 5-FdUMP and 5-FUTP is the formation of an intermediary metabolite, 5-fluorouridine monophosphate (5-FUMP), and it may only be produced after a series of catalysed enzymatic reactions (**Figure 2**). This can be circumvented by the ability of UPRT to convert 5FU directly to 5-fluorouridine monophosphate leading to more efficient production of antitumour metabolites, 5-FdUMP and 5-FUTP [90]. UPRT used in conjunction with CD and 5FC GDEPT was more effective than CD-GDEPT alone against colon cancer [91,92] glioma [93] or DU145 human PC cells [94] *in vitro* and in tumours grown *in vivo*. The use of the combination gene (as opposed to either gene singly) and markedly sensitises various cancer cells to low concentrations of 5FU [95]. A useful development was the elucidation of a method that allows magnetic resonance spectroscopy to be used to measure the conversion of 5FC to 5FU *in situ* [96].

The yeast CD is more efficient than the *E. coli* enzyme in converting 5FC to 5FU [97]. A combination of two yeast genes, *FCY1* and *FUR1*, encoding CD and UPRT, respectively, has been successfully used in an adenovirus to elicit GDEPT against mouse and human tumour cells [98] and a greater bystander effect was obtained than with either gene alone. In contrast to GCV and its derivatives, the 5FC and 5FU metabolites can penetrate tumour cells bypassive diffusion and expand the local toxic effect to neighbouring cells irrespective of the presence or absence of cellular connexins [98].

Apart from beneficial synergies with other GDEPTS (UPRT and HSV-tk), CD can sensitise cells to irradiation, making combinations of CD–GDEPT with irradiation effective in the clinic [40,41]. Several clinical trials for PC have been conducted using a combination of CD–GDEPT and HSV-tk–GDEPT with or without irradiation.

6.3 PNP and prodrugs

Although mammalian purine nucleoside phosphorylase (PNPs) exist, they differ fundamentally in sequence, structure and function from their bacterial counterparts [99]. Bacterial (but not mammalian) PNPs can cleave adenosine analogues to toxic adenine analogues. There are two advantages of using *E. coli* PNP for GDEPT against PC: PNP–GDEPT can kill cells independently of proliferation [100] for PC, where as few as 2% of the cells may be in S phase [39]; and the extensive bystander effects that can be achieved with this system [101]. The prodrug, fludarabine (arabinofuranosyl-2-fluoroadenine monophosphate), is converted to 2-fluoroadenine, which is nonphosphorylated and readily diffuses across cell membranes. *In vitro*, only 1% of the cells need to express *E. coli* PNP for total cell death to occur [101]. Strong bystander killing is seen in tumour xenografts and is dependent on the dose of both the toxic drug produced *in situ* and of the prodrug administered [99]. This *in vivo* tumour growth delay is enhanced when PNP–GDEPT is preceded by the pretreatment of mice with antibiotics to eliminate enteric flora encoding PNP enzymes [25]. Thus PNP–GDEPT provides a significant advantage over the HSV-tk system, in which phosphorylated toxic products require connexins [82] to spread to nearby cells, especially for the treatment of slow-growing PC.

Early studies with PNP used 6-methylpurine deoxyriboside as a prodrug [84]. This is converted by PNP to 6-methylpurine (6MP) [100]. Other groups have used fludarabine phosphate, which has the advantage of already being in the clinic as an antileukaemia agent [102]. Fludarabine is converted to 2 fluoroadenine (2FA), a base analogue that inhibits reactions involving ATP. Thus cycling and nonproliferating cells are killed by inhibition of protein and RNA syntheses [100,101]. Although conversion of fludarabine to 2FA is ~ 1000 times less than the cleavage of 6-methylpurine deoxyriboside to 6MP, 2FA is a more potent (~ 100-fold) cell growth inhibitor than 6MP [99,100]. Another advantage of this system is that the induction of apoptosis by PNP–fludarabine–GDEPT is independent of p53 status and the Fas/FasL pathway [103]; it worked well in both LNCaP (phenotypically wild-type p53 [104]) and PC-3 (p53-null [104]) cells [26]. Mutations of p53 have been shown to predispose to cancer progression when they occur as an early event in PC [105]; thus the use of PNP/fludarabine–GDEPT could provide a substantial advantage over other GDEPT systems for patients whose PCs express p53 mutations.

PNP–GDEPT has been shown to have greater efficiency against PC *in vitro* than HSV-tk–GDEPT or GCV-GDEPT when introduced under the same PSA promoter in otherwise identical human adenoviruses [47] and was also effective against human PCs grown in nude mice *in vivo* [84]. When delivered by an OAdV, PNP–GDEPT was highly effective against both androgen-dependent and HRPC human xenografts [26] or in an immunocompetent mouse model [106], and caused highly significant suppression of PC progression in immune-competent

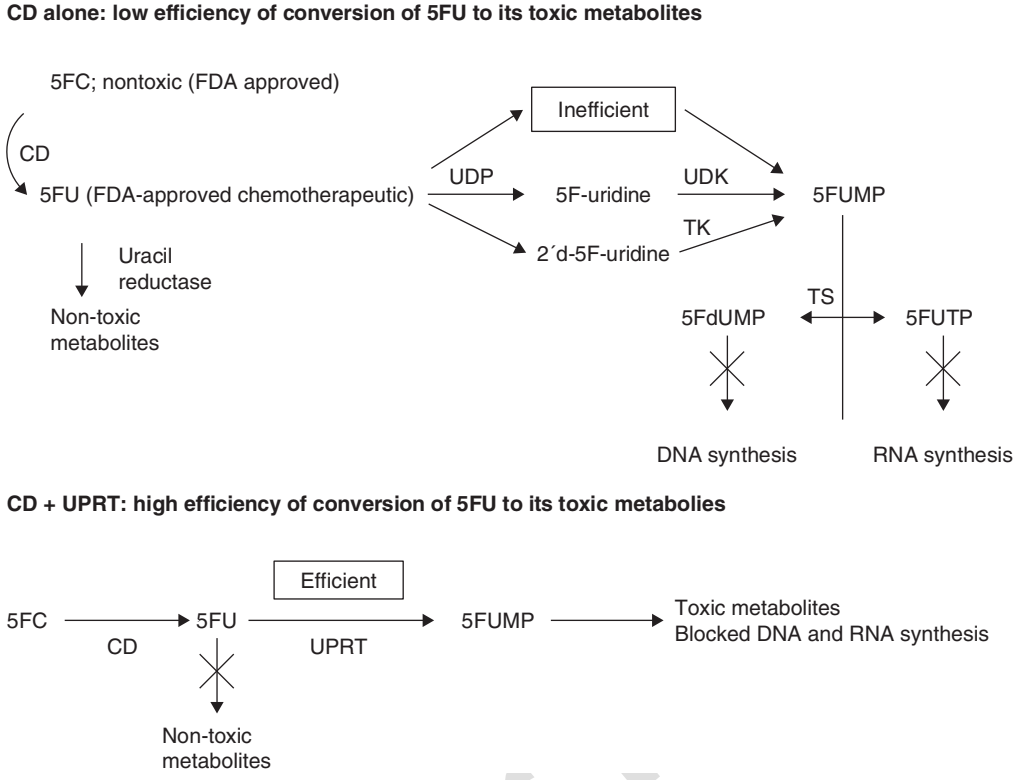

Figure 2. Synergy between CD and UPRT GDEPTs. Conversion of 5FU to its toxic metabolites is inefficient in mammalian cells as it involves a series of catalytic steps. This inefficiency also leads to its conversion to nontoxic metabolites. Combination of CD with UPRT circumvents this by directly converting 5FU to 5FUMP, thus bypassing the intermediate steps as shown.

5FA: 5-Fluorouracil; 5fC: 5-Fluorocytosine; 5FUTP: 5-Fluorouridine triphosphate; CD: Cytosine deaminase; dUMP: Deoxyuridine monophosphate; dTMP: Deoxythymidine monophosphate; GDEPT: Gene-directed enzyme prodrug therapy; UDK: Uridine kinase; UDP: Uridine phosphorylase; UMP: Uridine monophosphate; UPRT: Uracil phosphoribosyltransferase; TK: Thymidine kinase; TS: Thymidylate synthase.

transgenic adenocarcinoma of mouse prostate mice [107]. Furthermore, it was able to elicit a distant bystander effect, inhibiting the formation of lung metastases in immunocompetent mice when delivered into the prostate [108].

Although not yet tested in the clinic, PNP–GDEPT has great potential for treating local and metastatic PC and efforts aim to enhance its efficacy. Recently, crystallographic and computer modelling methods were used to redesign *E. coli* PNP to cleave new prodrug substrates, showing > 100-fold increase in efficiency compared with wild-type enzymes [109].

6.4 Nitroreductase and prodrugs

The *E. coli* nitroreductase (NTR) system uses alkylating agents as prodrugs that include dinitroaziridinylbenzamides, dinitrobenzamide mustards, 4-nitrobenzylcarbamates and nitroindolines. Most of the work has been carried out with the dinitroaziridinylbenzamide CB1954 (the relative merits of these prodrugs are discussed elsewhere [69]). The prodrug CB1954 (5-[azridin-1-yl]-2,4-dinitrobenzamide; a weak monofunctional alkylating agent) is converted by NTR to its 4-hydroxylamino derivative [110,111], which becomes a powerful bifunctional alkylating agent following acetylation via thioesters such as acetyl coenzyme A that forms DNA crosslinks in both cycling and noncycling cells, and has both local and distant bystander effects. The local bystander effects are related to both 2- and 4-hydroxylamine production; the corresponding dinitrobenzamide nitrogen mustard SN23862 is reduced by NTR to a single extracellular metabolite (the 2-amine), which has superior diffusion and cytoxicity than CB1954 metabolites [112]. The distant bystander effect may relate to induction of a range of stress proteins, including Hsp-25 and -70 [113] and is enhanced by coexpression of Hsp-70 [114]; Hsp-70 can shuttle cytosolic peptides into antigen-presenting cells for induction of antitumour immunity. Combination of NTR–CB1954 GDEPT with genes for murine GM-CSF has also been shown to increase the immune response generated by the GDEPT [115]. The NTR–CB1954 GDEPT has also been used to kill human umbilical vein endothelial cells (HUV-EC-C), resulting in bystander killing of nearby tumour cells in an *in vitro* model [116]. A Phase I clinical trial of CB1954 in cancer patients documented the pharmacokinetics and dosage, and a CRAd-expressing NTR (CTL102) has been successfully tested in xenografts, including PC [117]. Despite the promise, a separate study

reported dose-limiting hepatotoxicity from CB1954, possibly as the liver is capable of aerobic reductive bioactivation of CB1954 to cytotoxic metabolites [118]. Hence, the focus is on developing more effective and less toxic prodrugs using the crystal structure of NTR [119] to provide the basis for site-directed mutagenesis [117].

6.5 CYP and cyclophosphamide or analogues

Oxazaphosphorines, cyclophosphamide and ifosfamide are metabolised by liver CYP to therapeutically active cytotoxic nitrogen mustards that form crosslinks with DNA, leading to apoptosis or necrosis but their effective use is limited by host toxicity. CYP–GDEPT is used to overcome this through local drug delivery. Enhanced efficacy of CYP–prodrug therapy is achieved by coexpression of the flavoenzyme, NADPH–CYP reductase, by metronomic (antiangiogenic) scheduling of the prodrug, by localised delivery to the tumour, and by combination with antiapoptotic factors that slow the death of the CYP factory cells [120]. The advantages of this system are the substantial bystander effects engendered; the ability to use human genes; bypassing any immune response to the transgene itself; the use of well-established conventional chemotherapeutic prodrugs in a hypoxic tumour environment; and the potential to decrease systemic exposure to active drug metabolites by selective inhibition of hepatic CYP activity [120]. Newer prodrugs, such as glufosfamide (NSC 612567 and NSC 613060), do not require liver activation as they are activated by phosphodiesterase in plasma and other tissues [121], but their metabolism is affected by many factors associated with the drugs, the patients and drug transporters, that have a major impact on pharmacokinetics and toxicity. CYP 2C9 was found to sensitise various human PC cell lines to cyclophosphamide treatment *in vitro* [71]. Phase I and II clinical trials have been performed with promising results in breast and melanoma cancer patients when retroviral or encapsulated CYP expressing cells were used for delivery [122]. An important use of CYP–GDEPT is to sensitise hypoxic tumour cells to radiotherapy (see Section 7.1).

7. GDEPT in combination with other therapies

In the absence of a single modality cure for PC, the emphasis of current research is in evaluating useful synergies between GDEPT and different modalities recapitulating on their individual properties. **Table 1** summarises various GDEPTs and their synergies with other modalities.

7.1 Sensitisation to chemotherapy and radiation by GDEPT

Although some studies have clearly shown the therapeutic potential of synergies between different GDEPTs, studies assessing synergies between GDEPT and traditional chemotherapy are minimal. Recently, synergy between NTR–CB1954–GDEPT and 5FU chemotherapy was demonstrated against cancer cells [123]. In contrast, several studies have shown beneficial synergies between GDEPT and radiation. Strategies to increase radiation sensitivity involve targeting of hypoxic tissues with bioreductive drugs that are preferentially toxic to tumour cells in a hypoxic environment; for example, AQ4N is metabolised in hypoxic cells by CYPs to the cytotoxin AQ4, which causes DNA strand breaks and cell death. Enhancement of CYP levels may be obtained by GDEPT, thus increasing the response to radiation or cyclophosphamide [124]. Similarly, a combination of a prodrug, RSU1069, with a reduced radiotherapy dose and CYP reductase (P450R) cured xenografts *in vivo* [125]. Tirapazamine is also dependent on the cellular complement of reductases, including NADPH:P450R. Cowen *et al.* [126] have used an adenovirus incorporating a hypoxia-responsive element from the lactate dehydrogenase gene in a minimal SV40 promoter upstream of the cDNA for P450R to induce radiation sensitivity in a tumour model (not PC). Such methods increased tumour growth delay. Significant antitumour effects were also achieved with NTR–CB1954 when used with a hypoxia-inducible promoter [127].

Another strategy used clinically to sensitise patients with PC to irradiation is the use of replication-competent oncolytic virus (E1B-55K deleted; Ad5-CD/TK*rep*) to deliver a fusion gene comprising HSV-tk and CD to patients with local recurrence of PC after definitive radiation therapy [38] or with newly diagnosed, intermediate- to high-risk PC [39]. The Ad5-CD/TKrep virus was injected intraprostatically; 2 days later, patients received 5FC and GCV or valganciclovir respectively, for 1, 2 or 4 weeks, and conventional-dose three-dimensional conformal radiation therapy. A total of 7 out of 16 patients with local recurrence showed a decrease in serum PSA, and 2 patients were negative for PC 1 year later. There were no dose-limiting toxicities and therapeutic transgene expression persisted in the prostate for ~ 3 weeks post-CRAd injection [42]. PSA dropped to ≤ 0.5 ng/ml in 5 out of 10 patients not given androgen-deprivation therapy, showing great promise for this approach. A Phase I – II clinical study combining radiotherapy with HSV-tk/valganciclovir with or without hormonal therapy (the luteinising hormone-releasing agonist and antiandrogen flutamide for 14 months) was also performed in PC patients (arm C) [128]. All of the arm A (low-risk) patients (treated with HSV-tk–GDEPT plus irradiation) and arm B (high-risk) patients (who had radiotherapy and androgen ablation) had PSA biochemical control at last follow up (mean: 13.4 – 13.9 months), whereas 3 patients in arm C (stage D disease; pelvic lymph node involvement) who had the same treatment as in arm B plus additional radiotherapy to the lymphatics) had biochemical failure. Biopsies showed no evidence of cancer in 67% (18/27) of arm A at 6 weeks and in 100% (6/6) at 24 months after treatment and a high response was seen in arm B at similar time points. The use of GDEPT as an adjunct was considered as being worthwhile. Recently, a second-generation CRAd that delivers a mutant HSV-tk and a yeast CD (that are more catalytically efficient than bacterial

Table 1. Summary of GDEPTs and their synergies in the treatment of prostate cancer.

GDEPT	Prodrug/s	Mode of action of toxic metabolites	Intercellular transport of toxic metabolites	Beneficial synergies with
HSV-tk	Gancyclovir Acyclovir Penciclovir Valacyclovir	Block DNA synthesis, Inhibition of DNA polymerase*	Via gap junctions	Oncolytic Ad (Phase I/II clinical) CD (bacterial and yeast; Phase I/Iiclinical) CD + oncolytic Ad+ radiation (Phase I/II clinic) Cytokine Immunotherapy (IL12; preclinical)
CD	5FC 5FU	Block DNA and RNA synthesis	Gap junctions and diffusion	UPRT (bacterial and yeast; preclinical) HSV-tk (Phase I/II clinical) Radiation (Phase I/II clinical) Oncolytic Ad (Phase I/II clinical) HSV-tk+ oncolytic Ad + radiation ((Phase I/II clinical)
PNP	6-methylpurine deoxyriboside Fludarabine phosphate	Block DNA, RNA and protein synthesis	Passive diffusion	None published thus far‡
NTR	Dinitroaziridinylbenzamides (CB194)§ Dinitrobenzamide mustards 4-nitrobenzylcarbamates Nitroindolines	Alkylating agents; crosslink DNA in cycling and non cycling cells	Diffusion¶	Heat-shock protein (Hsp-70 and -25; preclinical) GM-CSF (preclinical) Oncolytic Ad (preclinical) Chemotherapy (5FU; *in vitro*)
Cytochrome P450	Oxazaphosphorines Cyclophosphamide Ifosfamide Glufosfamide	Nitrogen mustards; crosslink DNA cycling and noncycling cells	Diffusion	Radiation (preclinical) AQ4N + radiation (preclinical) Chemotherapy (AQ4N and tripazamine)

*Acyclovir and gancyclovir inhibit the DNA-polymerase activity via prevention of the elongation of new DNA strand leading to chain termination.‡Although no synergies using PNP GDEPT have been evaluated thus far, there are preclinical studies implicating the immune system in distant bystander effects suggesting the scope for synergies with immunotherapy. §CB1954 is the most commonly used drug of all. ¶Alternative prodrugs (SN23862) producing metabolites with superior diffusion capability to CB194 have been developed.

5FC: ; 5FU: 5-Fluorouracil; Ad: adenovirus ; CD: ; GDEPT: Gene-directed enzyme prodrug therapy; HSV-tk: Herpes simplex virus thymidine kinase; NTR: Nitroreductase; PNP: Purine nucleoside phosphorylase; UPRT: Uracil phosphoribosyltransferase;.

and wild-type counterparts) together with the adenovirus death protein has been developed [129]. This virus has shown greater tumour control in preclinical models of PC than the first virus [42] without any increase in toxicity when injected intraprostatically.

8. Immune reactions generated by GDEPT

Intraprostatic HSV-tk/GCV–GDEPT has been shown to generate an immune reaction that results in a decrease in the number of pseudometastasis in the lungs of immunocompetent mice after intravenous injection of the cell line of interest [45,46]. A distant bystander effect has also been demonstrated for other GDEPT systems, including PNP [108], NTR–CD1954 [114] and CD–UPRT/5FC [130]. These reactions involve T cells, macrophages and natural killer cells, and can be increased by the co-delivery of immune gene therapy such as co-expression of Hsp-70 [114], GM-CSF [115] or IL-12 [46]. Combination Ad–HSV-tk–GCV and Ad–IL-12 therapy prolonged the lifespan of the treated mice but did not achieve an increase in antimetastatic activity [131]. There have been three clinical trials conducted using HSV-tk–GCV or valacyclovir in patients with PC who: failed radiotherapy; had neoadjuvant gene therapy prior to prostactomy; or GDEPT plus radiotherapy for PC [132], which caused a statistically significant increase in circulating $CD8^+$ T cells 2 weeks post treatment; this was higher in men in trials B and C compared with A and was accompanied by an increase in activated $CD4^+$ T cells in men in trial C. These data indicate that systemic T-cell responses are generated in patients with PC after HSV-tk–GDEPT and that this response is further increased by the additional use of radiotherapy. In another trial conducted by the same group, 33 patients (PSA 10 or Gleason score 7) were treated with a combination of Ad5–HSV-tk/valacyclovir, radiation and androgen ablation [133]. The systemic T-cell responses were noted ≤ 12 months post-treatment and androgen ablation (2.3 years) did not have any effect on this immune response. It is anticipated that such immune responses may help to reduce micrometastases from PC and may ultimately lead to long-term cure when augmented further (through the use of immunostimulatory cytokines and other immunotherapies).

9. Conclusions

The authors have reviewed the progress and potential of various GDEPT systems for treating PC. As the prostate is stereotactically accessible and dispensable, PC is an ideal target for GDEPT and the localised delivery of drugs avoids toxicities associated with their systemic administration. Although numerous GDEPTs have been developed and evaluated to date, careful consideration of their properties (enzymes and prodrugs or toxic metabolites) would be useful in determining the optimal system/s (alone or in combination) for treating slow-growing cancers such as PC in all stages. Researchers have continuously designed new prodrugs with improved stability and increased efficacy. Although some of them show promise, none has shown a sufficient therapeutic index in the clinic. To maximise the benefits from GDEPT with minimal systemic toxicity, it needs to be delivered efficiently, sufficiently and in a tissue-specific manner; although nontoxic, nonviral vectors are not as efficient as viral vectors for *in situ* gene delivery. In this regard, oncolytic PC-targeted (transductionally and transcriptionally) adenoviral vectors have shown great promise. There is also a need to tap into systemic anticancer immune effects that are associated with GDEPTs by combining them with immunotherapy to target late-stage metastatic disease. Finally, an all-rounded approach will be crucial to achieving complete cure of this challenging disease. Synergies between different GDEPTs and other modalities, traditional and alternative, need to be explored to maximise therapeutic benefit with minimal toxicity.

10. Expert opinion

A plateau appears to have been reached in what can be achieved with conventional approaches such as surgery, radiation, chemotherapy and androgen ablation in men with advanced PC. There is a need to explore biologically rational novel treatments to improve outcomes in this highly malignant disease. With advances in gene technology and understanding of PC biology, cytoreductive gene therapy has emerged as a promising, viable adjuvant/alternative for treating cancer, especially PC; however, inefficient gene delivery poses a major hurdle to success. This was (in part) overcome by the discovery of GDEPT (HSV-tk) that demonstrated *in situ* amplification of gene expression-related cytotoxicity leading to the development of several GDEPT systems for assessment. The potential of GDEPT for cancer therapy relates to: localised administration of the toxic drug avoids the systemic toxicities associated with chemotherapy; prodrugs/drugs that are used are clinically tested anticancer agents with well-established pharmacokinetics and safety parameters; and amplification of *in situ* treatment-related targeted cytotoxicity via local and distant bystander effects. Crystallographic elucidation of enzyme conformations coupled with new technologies has led to new designer prodrug analogues to improve the efficacy, stability and safety of these systems. Despite substantial progress, none of these appears to engender sufficient therapeutic effects in the clinic. The focus now is to enhance their efficacy through: enhancing gene delivery; and beneficial synergies with other modalities; traditional as well as novel alternatives. With major advances in vector development, current research points to the use of viral vectors, especially adenoviral, as the best option for GDEPT delivery. The recent development of tissue-targeted oncolytic adenoviral vectors is a major step towards solving the issue of sufficient *in situ* gene delivery. In addition, the use of these lytic vectors leads to the death of selectively infected cancer cells, generates antitumour immune responses and shows synergies with traditional chemo- and radiation therapy, as clearly shown by recent clinical trials [40,41].

A focus on synergies between different GDEPTs and with traditional therapies would help to target the heterogenous population of cancer cells found in PC. Such synergies lead to a reduction of individual modality dose and hence less toxicity and increased quality of life for these patients. Alkylating agent-based GDEPTS (NTR and CYP) should be developed further, alone and with other GDEPTs or modalities to tackle drug resistance associated with standard chemotherapies. The ability of CYP–GDEPT to sensitise hypoxic tumour cells to radiation is especially appealing. GDEPT associated immune stimulation can be further augmented via combination with immunotherapy (e.g., use of immunostimulatory cytokines) to generate potent systemic long-term antitumour responses. This strategy is undergoing research by some groups, including the authors' own. Finally, future research for therapies against PC should aim to improve quality of life of HRPC patients and achieve effective therapeutic indices for all stages of PC. The cancer needs to be targeted on all fronts by combined modalities to achieve long-term cure. New research to target CRAds armed with GDEPT specifically to PCs in conjunction with chemo- or radiation therapy is needed to enhance safe GDEPT delivery to local and metastatic PC. The stringency of tumour-specific promoters and tissue tropism (transductional targeting) will play a major role in the targeting and safety of these vectors, especially for the treatment of metastatic PC. Finally, designer synergies between or among different types of GDEPTs, traditional therapies and/or other cytoreductive therapies such as immunotherapy need to be explored rigorously to maximise therapeutic effects and minimise toxicities.

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Targeted, Gene-directed Enzyme Prodrug Therapies to Tackle Diversity and Aggression of Late Stage Prostate Cancer

Aparajita Khatri and Pamela J. Russell

Summary: Late stage hormone refractory prostate cancer (HRPC) is presently incurable. Novel alternatives such as cytoreductive Gene Directed Enzyme Prodrug Therapy (GDEPT) offer great hope: The potential for in situ amplification of cytotoxicity due to GDEPT-associated "bystander effects" has special appeal for patients with prostate cancer, the prostate being dispensable. In this overview, recent developments in various GDEPT systems for treating prostate cancer are described. Research related to the enhancement of in situ GDEPT delivery and prostate cancer-targeting of viral vectors, is reviewed. The scope and progress of synergies between GDEPT and other treatment modalities, traditional and alternate, are discussed.

Prostate Cancer: The Problem!

Prostate cancer is the second highest killer from cancer in men. Prostate cancer is a slow growing tumor that requires androgenic hormones for growth/function at early stages. Surgery and radiation are first line therapies when prostate cancer is organ confined whilst androgen ablation is used for locally advanced or metastatic prostate cancer (Ryan and Small, 2005). Eventually, prostate cancer progresses from the androgen dependence (14-30 months) to hormone resistance, called Hormone Refractory Prostate Cancer (HRPC), for which there is no effective cure. Patients with HRPC may present with elevated prostate specific antigen (PSA) with or without metastases and radiologic (soft tissue and bone) and clinical (aggravated pain and urinary obstruction) progression. HRPC resists treatment; systemic chemotherapy only offers palliation with ~1 year median survival. In recent landmark trials, Docetaxel, a semisynthetic taxane, conferred 2 months survival benefit in 40% of HRPC patients, setting a new standard of care (Beer et al., 2001). Supportive care with bisphosphonates and radionuclides has reduced skeletal adverse events with better pain control.

Prostate cancer exhibits heterogeneity; no single modality can eradicate disease and lack of treatment specificity and high dosing requirements cause unwanted side effects that lower the quality of life. The current focus is to explore novel combinations using components that 1) maximize therapeutic benefit via targeting the heterogeneous prostate cancer cell populations and 2) minimize therapeutic doses of individual treatments to reduce associated toxicities and improve quality of life. Cancer cells live in a complex microenvironment involving interactions with stroma, endothelial cells, and the immune system. Components of combination

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regimens need careful selection/development to target not only heterogeneous tumor cells but also their microenvironment, and to augment systemic anticancer responses to prevent/target invasion and metastases.

Apart from traditional treatments, several alternative biological therapies are being explored, including cytoreductive therapies (suicide gene therapy, immunotherapy), antiangiogenesis therapy, vaccines to boost immune responses against prostate cancer, targeted antibody therapy, proteasome inhibition, and growth factor inhibition.

Gene-directed Enzyme Prodrug Therapy

With advances in understanding prostate cancer biology, tissue-targeted cytoreductive gene therapy such as Gene-Directed Enzyme Prodrug Therapy (GDEPT) offers a viable alternative/adjuvant therapy (Russell and Khatri, 2006; Kaliberov and Buchsbaum, 2006). Prostate cancer is ideal for GDEPT as the prostate is "dispensable" and stereotactically accessible for local (intraprostatic) intervention, avoiding normal tissue toxicity that occurs with systemic gene delivery. GDEPT offers a unique targeted approach with potential for amplification of in situ cytotoxic effects locally and systemically.

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Concept: deliver an enzyme, e.g., bacterial, yeast, or viral, that is absent or expressed at low concentrations (human) in normal tissue, directly into the tumor mass that can then convert a systemically administered "non-toxic prodrug" into a "toxic drug."

Components of GDEPT

1. Enzymes used in GDEPT catalyze specific reactions not catalyzed by endogenous enzymes, and should be delivered in sufficient amounts to effect prodrug conversion. The site of enzyme expression within the cell (cytoplasmic/nuclear or mitochondrial) is critical in determining efficiency of prodrug activation. For prostate cancer, those used include non-mammalian: thymidine kinase from Herpes Simplex virus (HSV-tk) or from Varicella zoster virus; *E. coli* enzymes: cytosine deaminase (CD), uracil phosphoribosyl transferase (UPRT), a fusion of CD and UPRT (CDUPRT), purine nucleoside phosphorylase (PNP), and nitroreductase; and the human enzyme: cytochrome P450 (CYP).

2. Prodrug for GDEPT should be 1) able to cross the cancer cell membrane for activation by GDEPT enzyme, 2) a good substrate, 3) of low cytotoxicity to normal tissues for systemic administration, and 4) able to form a drug that is highly cytotoxic, metabolically stable, and diffusible. Cellular retention of the prodrug and its toxic products must be long enough to achieve effective toxicity levels. New drugs with greater cellular retention for treating solid tumors such as prostate cancer are being developed.

Two types of prodrugs/drugs are used in different GDEPT systems (Denny, 2003): 1) antimetabolites, e.g., gancyclovir (GCV), have a complex pathway of activation and often induce resistance and 2) alkylating agents, e.g., cyclophosphamide, that act by cross-linking non-dividing and dividing cells so as to target a larger population of tumor cells.

Key Features of GDEPT

1. Localized delivery of the "toxic drug" avoids toxicity associated with systemic administration of such drugs.

2. Local bystander effect: Not all cancer cells need to express the transgene to be killed by the drug generated. The "toxic drug" produced spreads to surrounding cells leading to in situ amplification of cytotoxicity. This is strongly influenced by its size and metabolism. Small molecules like 2-fluoroadenine (a toxic drug formed in PNP-GDEPT) can diffuse passively into nearby cells, whereas highly phosphorylated toxic drugs, e.g., triphosphates of ganciclovir (from HSV-tk-GDEPT activity), require active transport or gap junc-

tions to effect toxicity in neighboring cells. The bystander effect of such drugs depends on the targeted cancer cell type.

3. Distant bystander effect: Local cell-killing in the tumor mass by GDEPT can stimulate systemic anticancer effects through the host immune system, providing the potential to decrease metastatic growth of cancer cells at distant sites. This involves natural killer (NK) cells, T cells, and macrophages.

GDEPT for Prostate Cancer

Several GDEPTs have been tried; others are under exploration. For prostate cancer, HSV/tk, CD, PNP, and CD together with UPRT and HSV/tk have had the most attention. Most use prodrugs already in the clinic with established pharmacokinetics and safety parameters; some have been tested in Phase I/II clinical trials alone or in combination with very promising results. Table 1 summarizes the advantages of various GDEPT systems for treating prostate cancer.

"New designer prodrug analogues to improve efficacy, stability, and safety are being developed through improved technology and a better understanding of enzyme conformation. With major advances in vector development, current research points to the use of viral vectors, especially adenoviral, as the best option for GDEPT delivery. Further, the use of GDEPT armed prostate cancer-targeted (transductionally and transcriptionally) oncolytic adenoviral vectors will enhance prostate cancer-specific therapeutic efficacy."

GDEPT Delivery

Despite in situ amplification, delivering enough enzyme into the cancer is the biggest hurdle to effective GDEPT clinically. Combination of a delivery system with GDEPT that targets both dividing and non-dividing cells would be ideal for prostate cancer, given that only 2% of the cells are dividing.

While both viral and non-viral delivery systems are being explored, viral vectors are more efficient for gene delivery in vitro and in vivo and hence the vectors of choice. Over 60% of gene therapy trials (GDEPT: 7.4%) have employed viral vectors, specifically, replication defective adeno- and related viruses (e.g., ovine atadenovirus, OAdV), because of their superior efficiency of gene delivery to both dividing and non-dividing cells in situ.

Oncolytic Adenoviruses Armed with GDEPT for Prostate Cancer Therapy

Despite the promise, efficacy of in vivo gene delivery by replication defective adenoviral vectors remains an issue. Recently developed targeted replication-competent oncolytic adenoviral vectors (conditionally replicating adenoviruses or CRAds) are genetically modified to replicate specifically in targeted cancer cells but not in normal cells. Not only is the therapeutic dose enhanced, but they also lead to the death of the selectively infected cancer cells via their oncolytic properties. The promise of these oncolytic vectors is obvious from their quick translation to the clinic and several clinical trials (including for prostate cancer) have shown no dose limiting toxicities associated with these vectors (http://www.cancer.gov/clinicaltrials).

CRAds and GDEPT

Given significant synergy with chemo- or radiotherapy, CRAds have been armed with GDEPT to further enhance their oncolytic properties and vice versa. Preclinical studies and clinical trials have shown that efficacies can be enhanced and toxicities reduced when using CRAds together with GDEPT (described later). This has important implications for treating solid tumors such as prostate cancer. Further, the immunogenic nature of these vectors may enhance GDEPT mediated anticancer immune responses with the potentially targeting systemic disease.

Targeting GDEPT to Prostate Cancer

GDEPT targeted to cancer cells/target tissue should be delivered with high efficiency avoiding gene expression in healthy tissues to achieve a superior therapeutic index. Thus far, adenoviral vectors have been given

directly into the patient's prostate, avoiding liver toxicity associated with systemic delivery. For disseminated disease, systemic delivery would be preferable. Researchers have achieved that via transductional and/or transcriptional targeting of vectors to cancer cells. This has been facilitated by the completion of human/mouse genome sequencing and a better understanding of tumor cell and viral vector biology.

Transcriptional Targeting

GDEPT expression can be transcriptionally regulated by using promoter/enhancers that are active only in prostate cells. Suitable promoters have been developed and optimized to specifically target prostate cancer, late stage HRPC, tumor microenvironments, bone stroma/metastases, or both (Table 2). Such promoters can also be used in CRAds to target viral replication to

Table 1. Summary of GDEPTs and Their Properties and Advantages

GDEPT	Prodrug/s	Mode of Action of Toxic Metabolites	Intercellular Transport of Toxic Metabolites	Features and Advantages
HSV-tk	Gancyclovir Acyclovir Penciclovir Valacyclovir	Block DNA synthesis, Inhibition of DNA polymerase[1]	Via gap junctions	Most commonly used GDEPT, tested in clinic Local bystander effects vary depending on GAP junction status of target cell Distant bystander effects involve immune system (Immunostimulatory) Application in in vivo imaging to monitor vector biodistribution and gene expression in clinical trials
CD	5 fluoro-cytosine (5FC) 5 fluoro-uridine (5FU)	Block DNA and RNA synthesis	Gap junctions and diffusion	Second most common, tested in clinic Local bystander effect Distant bystander effect With UPRT targets both dividing and non-dividing cells with greater local and distant bystander effects
PNP	6-Methyl-purine Fludarabine phosphate	Block DNA, RNA and protein synthesis	Passive diffusion	Local bystander effect Distant bystander effect Immunostimulatory Not tested in clinic More potent than HSV/tk
NTR	Dinitroaziridinyl-benzamides (CB194[2]) Dinitrobenzamide mustards 4-nitrobenzyl carbamates Nitroindolines	Alkylating agents; crosslink DNA in cycling and non cycling cells	Diffusion[3]	Potent local/distant bystander effects Involvement of immune system, stress proteins Established pharmacokinetics and drug dosage (Phase I clinical trial). Dose limiting hepatotoxicity due to aerobic reduction of drug to cytotoxic metabolites in liver
Cyto-chrome P450	Oxazaphosphorines Cyclophosphamide Ifosfamide Glufosfamide	Nitrogen mustards; crosslink DNA cycling and non-cycling cells	Diffusion	Potent local/distant bystander effects Ability to use human genes, bypassing any immune response; Well-established conventional prodrugs Effective in hypoxic tumor environment Sensitizes hypoxic cancer cells to radiotherapy

Adapted from Russell and Khatri, 2006.
1. Acyclovir and gancyclovir inhibit the DNA-polymerase activity via preventing elongation of new DNA strands leading to chain termination.
2. CB1954 is the most commonly used drug
3. Alternative prodrugs (SN23862) that produce metabolites with superior diffusion capability to CB194 have been developed.

prostate cancer. Solid tumors like prostate cancer contain regions deficient in oxygen (hypoxia) especially in association with metastases. Hypoxia inducible promoters, e.g., survivin, may enhance GDEPT expression in prostate cancer tumors.

Transductional Targeting

The structure of viral vectors can be modified to change their tropism, allowing entry via cancer cell receptors instead of their cognate receptor. This has the additional advantage of avoiding non-specific viral toxicity to tissues such as the liver. For adenoviral vectors, capsid proteins (Fibre Knob, pentons) used for viral entry, can be genetically modified to give different tissue specificities. Alternatively, the particles can be physically conjugated to cancer specific antibodies.

GDEPT in Combination with Other Therapies

While promising, GDEPT has not shown sufficient activity clinically. Several studies have shown synergies between molecular chemotherapies engendered by different GDEPTs, or by combining GDEPT with other therapeutics including chemotherapy or radiation (Table 3). Treatment of hypoxic tumor tissues with bioreductive drugs sensitizes these cells to radiation or chemotherapy.

An important clinical study used replication competent oncolytic adenovirus to deliver a fusion of HSV-tk and CD genes to patients with newly diagnosed, intermediate- to high-risk prostate cancer or with local recurrence of prostate cancer after definitive radiation therapy (Freytag et al., 2003). There were no dose limiting toxicities and 2 patients remained negative for prostate cancer one year later. PSA dropped to <0.5 ng/mL in 5/10 patients not given androgen-deprivation therapy, showing great promise for this approach. The same group combined radiotherapy with HSV-tk/valganciclovir with or without hormonal therapy in a Phase I-II trial that showed no evidence of cancer in 100% (6/6) of low-risk patients (treated with HSV-tk-GDEPT plus irradiation) at 2 years after treatment. A high response was seen in high-risk patients (radiotherapy and androgen ablation) at similar time points whereas 3 patients with Stage D disease (pelvic lymph node involvement with additional radiotherapy to the lymphatics) had biochemical failure. The use of GDEPT as an adjunct was considered worthwhile. These investigators are

Table 2. Prostate Cancer-specific Promoters

Promoters	Description	Specificity
PSA	Prostate specific antigen	Prostate cancer (PC); lungs
PSE	PSA with enhancer	PC
PSMA promoter	Prostate specific membrane antigen (PSMA) gene promoter	PC
PSES	Novel chimeric enhancers	Enhanced specificity to PC
PSME/Pb	Partial promoter from probasin (PB) plus enhancer from PSMA gene	PC and HRPC
PEPM	Combination of promoter/enhancer of PSMA gene	PC and HRPC
PSP94	Promoter/enhancer region from prostate secretory protein	PC and HRPC
Cav-1	Caveolin-1	Metastatic and HRPCs; tumor-associated endothelial cells
OC	Human osteocalcin promoter bony metastases of PC	Bone metastases
PSA+OC	Prostate specific antigen +osteocalcin promoter	Bone stroma and PC metastases

now investigating a second-generation CRAd that delivers a mutant HSV-tk and a yeast CD (more potent than bacterial CD) together with the adenovirus death protein (ADP) in the clinic.

Potentiation of GDEPT in Combination with Immunotherapy

Involvement of host immune responses was first implicated with HSV-tk-GDEPT associated reduction in prostate cancer metastases. Since then a distant bystander effect has also been demonstrated for other GDEPT systems, including PNP, NTR/CD1954, and CDUPRT/5FC. Both preclinical and clinical studies have demonstrated the involvement of T cells, macrophages, and NK cells in these effects. In clinical studies, a statistically significant increase in circulating cytotoxic T lymphocytes was reported in prostate cancer patients in 4 different trials after HSV-tk-GDEPT with/without radiation or androgen ablation. Such immune responses may help to reduce micrometastases from prostate cancer and when augmented further (through the use of immunostimulatory cytokines and other immunotherapies), may ultimately lead to long-term cure. In preclinical studies, combining GDEPT with immune gene therapy such as GM-CSF (granulocyte monocyte-colony stimulating factor), IL-12 and HSP-70, or in our studies, IL-12 plus IL-18, has significantly prolonged life span in treated mice.

Conclusions

No single modality can eradicate prostate cancer and late stage disease remains incurable. There is a clear need to explore novel biological treatments alone or together to improve outcomes for men with HRPC. The focus now is to enhance efficacy through 1) enhancing cancer specific gene delivery and 2) obtaining beneficial synergies with other modalities, both traditional and novel alternatives. The solution may lie in designing combination regimens that are more aggressive but carefully targeted to avoid systemic toxicities. A careful consideration of GDEPT properties (enzymes and prodrugs/toxic-metabolites) would help to optimize these systems (alone or in combination) for prostate cancer. New designer prodrug analogues to improve efficacy, stability, and safety are being developed through improved technology and a better understanding of enzyme conformation. With major advances in vector development, current research points to the use of viral vectors, especially adenoviral, as the best option for GDEPT delivery. Further, the use of GDEPT armed prostate cancer-targeted (transductionally and transcriptionally) oncolytic adenoviral vectors will enhance prostate cancer-specific therapeutic efficacy. The stringency of tumor-specific promoters and tissue tropism (transductional targeting) will play a major role in targeting and safety of these vectors, especially for treating metastatic prostate cancer. In addition, these lytic vectors kill selectively infected cancer cells, generat-

Table 3. GDEPT in Synergistic Combinations

GDEPT	Beneficial Synergies With
HSV-tk	Oncolytic Ad (Phase I/II) CD (bacterial and yeast) (Phase I/II) CD+Oncolytic Ad+ Radiation (Phase I/II) Cytokine Immunotherapy (IL12) (preclinical)
CD	UPRT (bacterial and yeast) (preclinical) HSV-tk (Phase I/II) Radiation (Phase I/II) Oncolytic Ad (Phase I/II) HSV-tk + oncolytic Ad + radiation ((Phase I/II)
PNP	None published thus far[1] Docetaxel[2]
NTR	Heat shock protein (HSP70, HSP25) (preclinical) GM-CSF (preclinical) Oncolytic Ad (preclinical) Chemotherapy (5FU) (in vitro)
Cytochrome P450	Radiation (preclinical) AQ4N+Radiation (preclinical) Chemotherapy (AQ4N, Tripazamine)

Adapted from Russell and Khatri, 2006.
1. Although not evaluated clinically, our preclinical studies using PNP GDEPT implicate the immune system in distant bystander effects suggesting scope for synergies with immunotherapy.
2. Unpublished data, our laboratory.
(http://www.cancer.gov/clinicaltrials)

ing anti-tumor immune responses and showing synergies with traditional chemo- and radiation therapy. An all rounded approach will be crucial to achieving complete cure of this complex disease. Designer synergies between different types of GDEPTs, cancer-specific CRAds, traditional therapies, and/or other cytoreductive therapies such as immunotherapy need to be explored rigorously to maximize therapeutic effects and minimize toxicities, especially against HRPC.

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Control/Tracking Number : 05-AB-5174-AACR
Activity : Abstract Submission
Current Date/Time : 11/28/2004 11:32:47 PM

Gene directed enzyme prodrug therapy using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase leads to a distant bystander effect in mouse models of prostate cancer.

Short Title:
CDUPRT-GDEPT for prostate cancer

Pamela J. Russell, *Aparajita Khatri*, *Bing Zhang*, *Eboney Doherty*, *Kim Ow*, *Jane Chapman*, *Rosetta Martiniello-Wilks*. Prince of Wales Hospital, Randwick, Australia, Prince of Wales Hospital, Sydney, Australia, Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using the fusion gene,cytosine deaminase uracil phosphoribosyl transferase (CDUPRT) for treating prostate cancer (PCa).
Objective: To test the efficacy of CDUPRT-GDEPT against RM1 mouse androgen-refractory PCa grown in C57BL/6 mice: RM1 cells were stably transfected with green fluorescence protein (GFP) and the fusion gene, CDUPRT, derived from *E coli* (RM1-GFP/CDUPRT). **CD/UPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, thus killing both dividing and non-dividing cells.** This is especially relevant to PCa, which is characterized by a low proportion of dividing cells.
Experimental Design: RM1 cells were stably transfected with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ (controls) using lipofectamine. Cells that highly expressed GFP were selected by flow cytometry and used for further study. Transgene CDUPRT expression in cell lysates from cells grown *in vitro* or after *in vivo* implantation of RM1-GFP/CDUPRT was assessed by enzymic conversion of its substrate using HPLC. To assess the local bystander effect of CDUPRT-GDEPT, C57BL/6 mice were implanted directly into the prostate with cell mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions; 4 days later, 5FC was given intraperitoneally (ip) for 13 days at 500mg/kg/mouse/day. Pseudo-metastases in the lungs were established by a tail vein injection (iv) of untransfected RM1 cells 4 days post intraprostatic implantation**.** Mice were euthanased on day 19, and prostate weight and volume, and lung weight and colony counts were assessed. Tumors, lymph nodes, spleens and lungs were frozen or fixed for immunohistochemistry.
Results: Intraprostatic RM1-GFP/CDUPRT tumors on treatment with 5FC for 13 days resulted in complete regression of the tumors. Injection of cell mixtures (RM1-GFP/CDUPRT + RM1-GFP) resulted in a local bystander effect when only 20% of

the cells were expressing the CDUPRT transgene. Interestingly, the lung colony counts indicated the presence of a distant bystander effect. The pseudo-metastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT.
Conclusions and future work: The CDUPRT GDEPT leads to a significant local and a distant bystander effect when used to treat androgen refractory RM1 tumors in mice. The role of the immune system in this distant bystander effect is currently under investigation.
Author Disclosure Block: P.J. Russell, None; **A. Khatri**, None; **B. Zhang**, None; **E. Doherty**, None; **K. Ow**, None; **J. Chapman**, None; **R. Martiniello-Wilks**, None.

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Australasian Gene Therapy Society, Melbourne, Oct, 2005

Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against RM1 prostate cancer in C57BL/6 mice.

Aparajita Khatri[1,2], *Pamela J. Russell*[1,2] , *Bing Zhang*[3], *Eboney Doherty*[1,2], *Kim Ow*[1], *Jane Chapman*[1,2], *Rosetta Martiniello-Wilks*[4].

[1]Prince of Wales Hospital, Randwick, Australia,[2] University Of New South Wales, Sydney, [3]University of Queensland, Brisbane, [4]Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using cytosine deaminase (CD) in combination with uracil phosphoribosyl transferase (UPRT) for treating prostate cancer (PCa). *CDUPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites, including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, resulting in the killing of both dividing and non-dividing cells*. This is especially relevant to slow growing PCa.
Andogen-independent mouse RM1 cells were stably transformed with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ genes (controls). CDUPRT expression in cell lysates from RM1-GFP/CDUPRT cells/tumors was confirmed by estimation of enzymic conversion of its substrate, 5FC to 5FU using HPLC. Treatment of C57BL/6 mice bearing intraprostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. Further, intraprostatic implantations with mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions in C57BL/6 mice resulted in a '***local bystander effect',*** even though only 20% of the cells were expressing the transgene. To determine if there was any distant bystander effect, pseudometastases in the lungs were established and the lung colony counts at necroscopy (day 19) indicated the presence of a '***distant bystander effect***'. Indeed, the pseudometastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. ***This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT.*** Furthermore, immunohistochemical evaluation of the GDEPT showed an increase in immune cell infiltration by $CD4^{+}$ T cells, macrophages and natural killer cells. There was increased tumor necrosis and apoptosis and a decrease in tumor vascularity after GDEPT. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate tumors via mechanisms involving necrosis and apoptosis, accompanied by strong infiltration of immune cells in the prostate tumors. The latter may be associated with the decrease in lung pseudometastases.

AACR 2007

Broadening of transgenic adenocarcinoma of the mouse prostate (TRAMP) model to represent late stage hormone refractory cancer.

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Short Title: HRPC TRAMP cells for preclinical studies

Currently there are no preclinical immunocompetent mouse models that adequately represent all stages of prostate cancer (PC). Development of such a model would be ideal to evaluate potential therapies, especially for late stage PC. Although, the best characterized transgenic TRAMP mouse model closely mimics PC as it occurs in humans, the timing of disease incidence and progression makes it logistically difficult to conduct experiments 1. Synchronously and 2. Economically (in terms of time and money).

Objective: To develop and characterize androgen-independent TRAMP sublines to represent late stages of prostate cancer progression in TRAMP transgenic model.

Methods: Three androgen independent sublines were derived from androgen sensitive TRAMP C1 (TC1) and TRAMP C2 (TC2) parental cells *in vitro* by dihydrotestosterone ***(TC1-T5 and TC2-T5)*** deprivation and *in vivo* from TC1 tumours that grew in TRAMP female mice ***(TC1-F1)***. The *in vitro* growth rate of the sublines was determined by trypan blue exclusion. The epithelial origin (cytokeratin) and metastaic potential (E-Cadherin) were evaluated using immunohistochemical analyses. Androgen receptor status was assessed through quantitative real time PCR, western-blotting and immunohistochemistry. The *in vitro* tumorigenicity and invasive potential *in vitro* were measured by their ability to form soft agar colonies and by invasion through matrigel, respectively. Real time quantitative PCR was used to assess the status of proteases (EMMPRIN and MMP9) implicated in tumor invasion and metastases. Androgen-independent growth of subcutaneous tumors was assessed in castrated and sham castrated C57BL/6 mice.

Results: Three androgen independent (AI) derivative sublines were established from androgen-sensitive TC1 & TC2 cell lines, TC1-T5, TC1-F1 and TC2-T5. All sublines expressed cytokeratin reconfirming their epithelial characteristics. In comparison to the parental cells, the sublines are 1. faster growing 2. more invasive and 3. androgen-independent *in vitro* and *in vivo*. Notably, androgen receptor is down-regulated in all three, with no expression in 2 lines, and they each demonstrate reduced expression of E-Cadherin; a transmembrane glycoprotein mostly lost in the advanced stages of cancer. PCR analysis showed upregulation of mRNA levels for both EMMPRIN and MMP9 in TC1 but not TC2 sublines.

Conclusions: The derived sublines are androgen-independent and show an increased tumorigenic and invasive phenotype. We have successfully expanded the TRAMP model to encompass late stage prostate cancer. This will be of great value in generating a more

broadly based and hence more clinically relevant assessment of preventative or therapeutic regimens for treatment of PC.

Title: Combined Gene Therapy with Cytosine Deaminase plus Uracil Phosphoribosyl Transferase and immunostimulatory IL12 and IL18 Cytokines for Treating Prostate Cancer in C57BL/6 mice.

Aparajita Khatri[1, 2], Yasmin Husaini[1,2], Kim Ow[1], Jane Chapman[4], Lara Perryman[1,2], Pamela J. Russell[1,2].

Short Title: Suicide Gene + Cytokine therapy & Cancer

We previously found ***local & distant bystander effects using*** cytosine deaminase (CD)+uracil phosphoribosyl transferase (UPRT) mediated gene directed enzyme prodrug therapy (CDUPRT-GDEPT) to treat mouse RM1 hormone-refractory prostate cancers (HRPC) in immunocompetent C57BL/6 mice. Treatment induced dose-dependent tumor infiltration by $CD4^+$ T cells, macrophages and NK cells, suggesting potential for synergy with immunotherapy. We describe using immuno-stimulatory interleukins IL12, IL18 of proven anti-tumorigenic potential (enhanced when combined), plus GDEPT.

Objective: To test efficacy of CDUPRT-GDEPT plus IL12+IL18 cytokine treatment against RM1 cells *in vivo*.

Experimental Design: RM1 cells or stable transformants that express GFP/CDUPRT (RM1CDUPRT) were used to generate intraprostatic (iprost) RM1 or RM1CDUPRT tumors or RM1 lung pseudometastases in C57BL/6 mice (intravenously, iv). To assess cytokine effects, mice with iprost RM1 tumors were injected with Adenoviruses expressing murine IL12 (AdmIL12) and/or murine IL18 (AdmIL18) on day 5; on day 6 RM1 cells were given iv. Mice were euthanased on day 17, and prostate weight/volume, and lung colony counts assessed. Tissues were frozen/fixed for immunohistochemistry. Mouse serum cytokine profiles were analyzed by Luminex technology. To assess combination of CDUPRT-GDEPT with cytokines, RM1CDUPRT cells were implanted iprost; 5 days later, AdmIL12 and/or AdmIL18 were given iprost followed by intraperitoneal 5-Fluorocytosine injections for 11 days. To assess "remote" effects, mice were given RM1 cells iv on day 6. Necropsy protocol was as above. Survival of mice on different treatments was also assessed.

Results: Unlike IL12 or IL18 alone, their combination caused significant reduction in local PC growth with clear synergy in reducing RM1 lung colonies to near negligible. Serum cytokine analysis showed significant effects: increases in Th1 IFN γ and IL18 and reduction in Th2 IL4 and IL10 levels in the IL12+IL18 group. Combining CDUPRT-GDEPT with IL12+IL18 led to further growth reduction of local PCs and lung colonies compared with individual therapies. There was a clear survival advantage with 28.7% of mice alive on day 33, despite suppression of serum cytokine levels compared with controls.

***Conclusions*:** Combined CDUPRT-GDEPT with mIL12+mIL18 therapy led to significantly reduced growth of local/remote deposits of HRPC RM1 tumors and survival advantage in mice, despite their immunosuppressed state.

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Manuscript Draft

Manuscript Number: JIM-D-07-00048

Title: Murine CTLL-2 cells respond to mIL12: Prospects for developing an alternative bioassay for measurement of murine cytokines IL12 and IL18.

Article Type: Research Report

Section/Category:

Keywords: CTLL-2; Bioassays; IL12; IL18; Synergy.

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Manuscript Region of Origin:

Abstract: Cell line based bioassays are becoming increasingly popular for assessment of biological activities of cytokines primarily because these are easy to perform and are not subject to donor variation. A well characterised cell line with world wide availability would further minimise the inter-assay variations. C57Bl/6 mice derived T cell line; CTLL-2 fits this criterion. We explored the potential of CTLL-2 cells to develop a bioassay to detection of murine (m) IL12 and mIL18. Both cytokines have shown significant activity against a number of cancers and importantly, act synergistically via mutual upregulation of each others receptors.
The preliminary flow cytometric analyses of immuno-stained CTLL-2 cells showed that ~65% expressed mIL12 and ~5% expressed mIL18 receptors suggesting that these may respond to mIL12. As predicted, cells incubated with different doses of mIL12 or mIL18 for 72h were responsive to mIL12 and not to mIL18. However, when pre-treated with mIL12 for 24h prior to incubation with mIL18, there was significant enhancement in response. The sensitivity of the response was comparable to that obtained using the conventional splenocyte-based IFN □ release assay. The cytokine specificity of the response was proven unequivocally when significant reduction in CTLL-2 response was observed in the presence of the relevant neutralising antibodies. Finally, we could successfully detect lowest doses of ~0.1pg/ml mIL12 or 40pg/mL of mIL18 in cell supernatants in a cytokine specific manner, which is lower than the resting levels of these cytokines in mouse sera. Again the sensitivity was comparable to that observed in the conventional IFN □ release assay. Hence, we have demonstrated the potential of
CTLL-2-based bioassay to accurately and reproducibly detect biologically active mIL12 and mIL18 in biological samples.

However, when pre-treated with mIL12 for 24h prior to incubation with mIL18, there was significant enhancement in response. The sensitivity of the response was comparable to that obtained using the conventional splenocyte-based IFN □ release assay. The cytokine specificity of the response was proven unequivocally when significant reduction in CTLL-2 response was observed in the presence of the relevant neutralising antibodies. Finally, we could successfully detect lowest doses of ~0.1pg/ml mIL12 or 40pg/mL of mIL18 in cell supernatants in a cytokine specific manner, which is lower than the resting levels of these cytokines in mouse sera. Again the sensitivity was comparable to that observed in the conventional IFN □ release assay. Hence, we have demonstrated the potential of
CTLL-2-based bioassay to accurately and reproducibly detect biologically active mIL12 and mIL18 in biological samples.

5th February 2007

Journal of Immunological Methods

Editorial Office
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Dear Editor/s,

I enclose a paper entitled: Murine CTLL-2 cells respond to mIL12: Prospects for developing an alternative bioassay for measurement of murine cytokines IL12 and IL18. by Aparajita Khatri, Yasmin Husaini and Pamela J Russell.

In this study, we have explored the potential of a murine cytotoxic T cell line, CTLL-2 cells, for developing a functional assay to detect biological potencies of murine cytokines, IL12 and IL18. This paper shows for the first time that CTLL-2 cells have murine IL12 receptors and that the cells are responsive to the presence of biologically active mIL12. The synergy between the two cytokines was used as the basis for further exploring the potential of these cells for assaying biologically active mIL18. We found that the cells respond to mIL18 when pretreated with mIL12. The detection was as sensitive and specific as the conventional mouse splenocyte based IFNγ release assay. Importantly, the response was reproducible. Hence this study for the first time demonstrates that CTLL-2 cells respond to mIL12 and mIL12/mIL18. These data will provide a baseline to develop an accurate, sensitive and reproducible CTLL-2-based bioassay to detect biologically active mIL12 and mIL18 in biological samples.
Development of this bioassay would have the following advantages: 1. minimal inherent interassay variations as CTLL-2 cells are available world wide (available in most laboratories including the non immunology laboratories like ours) and make up at the moment the best characterised murine T cell line. 2. Properly developed, this assay would potentially replace the commonly used IFNγ release assay for mIL12 and mIL18, avoiding the use of animals and hence donor variation. The assay would provide a cheaper and reliable alternative easily available to all researchers. We believe that this system has potential benefits for detection of functional mIL12 and mIL18 cytokines in biological fluids in preclinical studies especially those utilising the anticancer properties of the two cytokines together.
Each author has contributed to the work in this paper and is happy for its publication. We hope that this paper will be publishable in Journal of Immunological Methods and look forward to your timely review.

With kind regards,
Yours sincerely,

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Manuscript Draft

Title: Murine CTLL-2 cells respond to mIL12: Prospects for developing an alternative bioassay for measurement of murine cytokines IL12 and IL18.

Aparajita Khatri[1,2*], Yasmin Husaini[1,2,3], Pamela J Russell[1,2]

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Article Type: Regular Article

Order of Authors: Aparajita Khatri, Yasmin Husaini, Pamela J Russell.

Running Title: Potential of CTLL-2 cells for assaying biological activities of murine IL12 and IL18 cytokines.

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Abbreviations Used: CTLL-2: Interleukin-2 responsive cytotoxic T lymphocyte, WST-1: (4-[3-(-4-Iodophenyl)-2-(4-nitrophenyl0-2H-5-tetrazolio]-1,3-benzene disulphonate] , FCS: Foetal calf serum, PE: Phycoerythrocin, ATCC: American type culture collection, [^{3}H]TdR: tritiated thymidine, MTT: [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], TGFβ: Transforming growth factor beta, GM-CSF: Granulocyte macrophage colony stimulating factor

Abstract:

Murine CTLL-2 cells respond to mIL12: prospects for developing an alternative bioassay for measurement of murine cytokines IL12 and IL18.

Cell line based bioassays are becoming increasingly popular for assessment of biological activities of cytokines primarily because these are easy to perform and are not subject to donor variation. A well characterised cell line with world wide availability would further minimise the inter-assay variations. C57BL/6 mice derived T cell line; CTLL-2 fits this criterion. We explored the potential of CTLL-2 cells to develop a bioassay to detection of murine (m) IL12 and mIL18. Both cytokines have shown significant activity against a number of cancers and importantly, act synergistically via mutual upregulation of each other's receptors.

The preliminary flow cytometric analyses of immuno-stained CTLL-2 cells showed that ~65% expressed mIL12 and ~5% expressed mIL18 receptors suggesting that these may respond to mIL12. As predicted, cells incubated with different doses of mIL12 or mIL18 for 72 h were responsive to mIL12 and not to mIL18. However, when pre-treated with mIL12 for 24 h prior to incubation with mIL18, there was significant enhancement in response. The sensitivity of the response was comparable to that obtained using the conventional splenocyte-based IFNγ release assay. The cytokine specificity of the response was proven unequivocally when significant reduction in CTLL-2 response was observed in the presence of the relevant neutralising antibodies. Finally, we could successfully detect lowest doses of ~0.1 pg/ml mIL12 or 40 pg/mL of mIL18 in cell supernatants in a cytokine specific manner, which is lower than the resting levels of these cytokines in mouse sera. Again the sensitivity was comparable to that observed in the conventional IFNγ release assay. Hence, we have demonstrated the potential of

CTLL-2-based bioassay to detect biologically active mIL12 and mIL18 in biological samples accurately and reproducibly.

Keywords: CTLL-2, Bioassays, IL12, IL18, synergy

INTRODUCTION:

Research in the cytokine field has increased rapidly because not only are cytokines involved in the production, maintenance and function of the haematopoietic/ immune system, but several have proved successful in the clinic as biotherapeutic agents in the treatment of a range of diseases (infectious, autoimmune and cancer) (H.Ibelgaufts,2002 ; Dinarello et al., 1989; Hirano et al., 1990; Tracey and Cerami, 1993; A.Meager, 2004; Dinarello, 2005). In particular IL12 and IL18 are Th1 type immunostimulatory cytokines that have shown therapeutic potential for treating various types of cancers (reviewed in (Liebau et al., 2002; Tatsumi et al., 2003; Yamanaka et al., 2003; Liebau et al., 2004; Li et al., 2005). Importantly, IL12 and IL18 synergize with each other by upregulating each other's receptors. IL-12 can upregulate the production of the IL-18 receptor α (IL18Rα) chain on Th1 cells (Ahn et al., 1997) whilst IL-18 up regulates the IL12Rβ2 chain receptor (Chang et al., 2000). This shared upregulation of receptors provides a positive feedback mechanism allowing these cytokines to act synergistically. This synergism has been particularly effective for treating various cancers with significantly higher therapeutic effects than when either cytokine is used alone. As a result several groups (including ours) are evaluating the two cytokines in the treatment of cancers. An accurate measurement of cytokine activity in biological fluids and laboratory samples is a necessary aspect of that research. Two types of assays can be used- Immunoassays and Bioassays. Immunoassays measure both active and inactive cytokine while bioassay typically measure the biological activity of specific cytokine/s which is more relevant to the *in vivo* action of that cytokine (Mire-Sluis et al., 1995a; Mire-Sluis et al., 1995b). Generally, bioassays use purified haematopoietic cells (from blood or bone marrow) or continuous cell lines (reviewed in (Mire-Sluis et al., 1995b)). While purified haematopoietic cell-based cytokine bioassays are sensitive and provide a quantitative estimate of cytokine activity, these assays require i) animal donors and hence ii) are subject to donor variation, which makes inter-assay comparisons difficult to interpret. In addition, purified cell

preparations are often contaminated with other immunological cell types, which can skew the results and add to the inter-assay variability. Cell line based bioassays are not subject to donor variation as the cell lines are monoclonal continuously growing cultures, and there is no tedious purification of the cells involved as is the case with *in vivo* cell sources. Cytokine responsive cell line based bioassays are more specific and easier to perform with minimal inter-assay variability. One important feature of current research is the mounting emphasis on minimising animal usage for experimental purposes. The cell line-based bioassays assays have the additional advantage that they address the ethical need to minimise the numbers of experimental animals.

There are established bioassays for measurement of the biological activities of murine IL12 (mIL12) and IL18 (mIL18), based on IFNγ production by purified T cells from mouse splenocytes: these, however, require animal donors. There are cell line-based bioassays employing 2D6 cells (Maruo et al., 1997) for murine IL12 and Human myelomonocytic cell line KG-1 transfected with mIL18 receptor for mIL18 bioassay (Taniguchi et al., 1998), however, these cell lines are not easily available or are very well characterised.

A prerequisite for consistent performance of a bioassay is the reliability of the indicator cell line: 1.identical cultures should be available worldwide to potentially minimise discrepancies in the data obtained 2. often the inherent variability observed with cell line based bioassays is due to heterogeneity of the biological samples in terms of their source and constituent factors. Multiple growth factors can have multiple effects. The response of cell lines used for such assays can vary depending on interactions of cells with the different factors. Hence, a well characterised cell line with known responses to a number of these factors would contribute to a better design and minimise variations and enhance reproducibility. Multiple growth factors can have multiple effects. The response of cell lines used for such assays can vary depending on interactions of cells with different factors. Hence a well characterised cell line with known responses to a number of these factors would contribute to a better design and minimise variations and enhance reproducibility.

The CTLL-2 cell-line, a cytotoxic T cell line of mouse origin derived from C57BL/6 inbred mice ($H\text{-}2^b$) (Gillis and Smith, 1977; Baker et al., 1979) fits this criterion. It was developed in 1977, is well characterised and available in most immunological and other laboratories (including ATCC). In this study, we explored the potential of the CTLL-2 cell line for developing a cell line-based bioassay as a convenient and novel alternative to the existing assays for the detection of bioactive mIL12 and mIL18. We show that the response of CTLL-2 cells to the two cytokines is specific and reproducible and has the potential to be developed more stringently as a novel and more convenient alternative to existing bioassays.

MATERIALS AND METHODS:

1.Cell lines: CTLL2 cells (obtained from S Schibeci at Dr P Williamson's laboratory, The Millennium Institute, Westmead Hospital, Sydney; This cell line was originally obtained from ATCC) are derived from C57BL/6 mice and grow indefinitely in culture in the presence of murine IL2 (mIL2) (Roche Molecular Biochemicals, Switzerland) (Gillis and Smith, 1977). These cells were routinely cultured in DMEM containing 10% fetal calf serum, 20 mM glutamine, 0.039 mM 2-mercaptoethanol (Sigma, Missouri, USA) and 11mM Na-pyruvate (Sigma, Missouri, USA) and 20units(U)/mL of mIL2. 293A cells (Invitrogen, CA, USA) cultures were grown in DMEM, 10% fetal calf serum and 20 mM glutamine.

2. Antibodies and recombinant cytokines: Recombinant murine IL12 (R&D systems, Minneapolis, USA) and mIL18 (Medical and Biological Laboratories Co., LTD, Japan) were used. The following antibodies were used for different experiments: Anti-mouse IL12 (αmIL12, polyclonal goat IgG, R&D systems, Minneapolis, USA), Anti-mouse IL18 (αmIL18, Medical and Biological Laboratories Woburn, MA, USA), biotinylated anti-mIL12 receptor antibody (mouse IgG2a, BD biosciences, Pharmingen, USA), biotin-conjugated mouse IgG2a isotype control (Pharmingen, BD biosciences, USA), streptavidin-PE (BD Pharmingen, USA), anti-mIL18 receptor antibody (Goat IgG, R&D systems, Minneapolis, USA), normal goat IgG (isotype control for

mIL18R antibody, Vector laboratories, CA, USA), donkey anti-goat-FITC (IgG, Chemicon, USA) and anti-CD3-FITC (BD Pharmingen, USA).

3. Staining of CTLL-2 cells for evaluation of mIL12 and mIL18 receptors: Cells ($2x10^5$/tube) were washed twice in Phosphate buffered saline (PBS) containing 10% fetal calf serum (PBS/FCS), then incubated with the primary biotinylated anti-mIL12 receptor antibody (0.25µg/tube) or anti-mIL18 receptor antibody (1µg/tube) for 30 mins on ice. This was followed by two washes with PBS/FCS and incubation with the secondary antibodies, streptavidin-PE (1µg/tube) or anti goat-FITC (1µg/tube) for anti-mIL12 receptor or anti-mIL18 receptor staining, respectively, for 30 mins on ice. For negative controls, cells were incubated with equal amounts of isotype control antibodies, IgG2a-biotin (for mIL12R) and goat IgG (for mIL18 R) or secondary antibody alone. After staining, cells were washed and resuspended in PBS for analysis using a fluorescence based cell scanner (FACscan, BD) and the software "Cell Quest". Dead cells were excluded from analysis via propidium iodide (Sigma, Missouri, USA) staining.

4. Proliferation assay for CTLL2 cells: CTLL2 cells ($5x10^3$/well) were seeded in a 96-well plate and cell proliferation was measured at 24, 48 and 72 h at different concentrations (50-0.19 units/mL) of IL2 (Roche, USA). The cell proliferation and viability of the cells was measured at each time point using a calorimetric assay, based on the cleavage of the tetrazolium salt (4-[3-(-4-Iodophenyl)-2-(4-nitrophenyl0-2H-5-tetrazolio]-1,3-benzene disulphonate] (WST-1) by mitochondrial dehydrogenase in viable cells (according to the manufacturer's protocol, Roche Diagnostic GmbH, Applied science, Germany)**.** At each time point, cells were incubated for 4 h (determined after optimisation using CTLL-2 cells) with WST1 (10 µL of supplied reagent/100 µL) followed by measurement of absorbance at 450nM using a micro-plate reader (Sunrise, TECAN, Austria, GmbH) and "Magellan" software. For tritiated thymidine incorporation experiments, $2x10^4$ CTLL-2 cells (in 200 µL volume) were treated with different doses of IL2 and 24 h later 0.5 µci of tritiated thymidine was added to each well. After 8h incubation the cells were harvested on to a silica filter

using an automated cell harvester. The membrane was dried and the counts were measured using the soft ware 'Top Count'.

5. Detection of Bioactivity of cytokines using the CTLL-2 cells: CTLL-2 cells were tested for their proliferative responses after 72 h incubation with different doses of mIL12 and/or mIL18 (either recombinant protein or unknown quantity in laboratory samples), using the WST-1 based assay as described above. The protein content of the supernatants from mIL12 or mIL18 producing cells was determined using the standard BCA assay (Pierce, Rockford, IL, USA). The quantity of secreted cytokines was determined using the relevant ELISA kits (OptEIA^{TM} Mouse IL12 (P70) or IL18 ELISA set, BD Pharmingen, USA).

6. Detection of bioactivity of the cytokines using mouse splenocytes: The functional activity of the recombinant mIL12 and mIL18 cytokines was evaluated by their ability to induce IFNγ production from activated T cells. Spleens from C57Bl/6 mice were stored on ice until splenocytes were extraction (no more than 1 h) by teasing the chopped mouse spleen through a 100 micron metal sieve with the aid of a syringe plunger. Cells were harvested, washed twice in PBS containing 5% FCS (FACSWASH), then T cells were isolated using αCD3 antibody and magnetic bead based MACS anti-FITC beads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the supplier's instructions. Briefly, 10^8 cells suspended in 1 mL of FACSWASH were incubated with 30 µL of ∝CD3-FITC primary antibody for 1h on ice. The cells were washed three times in FACSWASH and incubated with 100 µL of MACS anti-FITC beads in 1 mL of FACSWASH for 15 mins on ice. These were then washed thrice, resuspended in 10 mL of FACSWASH and applied to the LS separation columns under magnetic fields. Captured cells were eluted in the absence of the magnetic fields and collected as a single T cell fraction, the purity of which was analysed using flow cytometry (we routinely obtained a purity of ~85-95%). In general, 2-5X10^5 T cells were seeded in 96 well culture plates in complete DMEM media and subjected to different cytokine treatments. At

72 h post treatment, the supernatants (100 μL) harvested from splenocytes cultured in different treatments were analysed for IFNγ release using ELISA based assays (BD Pharmingen, USA).

7. Data analysis

A one-way analysis of variance (ANOVA) was performed (GraphPad PRISM V4) if the data in multiple groups were normally distributed. A Tukey's post-test was performed if the ANOVA indicated a significant difference ($p<0.05$) between treatments. To compare data in two groups, paired T test analysis were performed and a 'p' value >0.05 was considered insignificant.

RESULTS:

Our primary aim was to assess CTTL-2 cells for developing an alternative cell line-based bioassay for detection of biologically active mIL12 or mIL18 in biological samples. This assay should 1) be easy to perform, 2) be of comparable, if not greater, sensitivity to exiting assays, 3) utilise a cell line that is easily available and well characterised and 4) be reproducible. As discussed earlier, we chose CTLL-2 cell line for this study because i) it is of murine origin and can be maintained in culture using both human and mouse IL2, ii) it is the most exhaustively characterised murine T cell line to date, and iii) it is commonly available in most immunology and other laboratories (including ATCC). All experiments were repeated at least three times and data from representative experiments are shown in the figures.

1. CTLL-2 cells express mIL12 and/or mIL18 receptors: Initially we assessed the suitability of these cells for development of functional assays for mIL12 and mIL18 by analysing them for the presence of mIL12 or mIL18 receptors using flow cytometric analyses of the immunostained cells. The data obtained showed that at least 65% of CTLL2 cell population have mIL12 receptors (Figure 1, Panel A and B). However, when CTLL-2 cells were immunostained with ∝mIL18 receptor antibody, only ~5% of the cells had mIL18 receptors (Figure I, Panel B). These data suggested that

CTLL-2 cells might be responsive to the stimulatory action of the cytokine mIL12, but the responses to mIL18 cytokine may be minimal. Given that upregulation of each other's receptors may be a mechanism to explain the synergy between the two cytokines (Ahn et al., 1997; Chang et al., 2000), we hypothesised that a CTLL-2-based bioassay could be developed to detect functional mIL18 by using the two cytokines together.

2. <u>The sensitivity of the new WST-1-based analysis of proliferation of CTLL2 cells is similar to that observed with the traditional thymidine incorporation method</u>: Since proliferation and anti-proliferation based assays are the most commonly used parameters for measuring cytokine activity, we chose to detect the level of cytokines through their ability to stimulate the proliferation of the CTLL2 cells. The most frequently used method involves measuring DNA synthesis as an estimate of cell proliferation and generally determines the amount of tritiated thymidine incorporated into DNA. While this method is automated and sensitive, it requires an elaborate set-up and the facilities to handle radioactive materials as well as their disposal. Alternative non-radioactive methods involve the measurement of the cellular metabolism to evaluate cell proliferation. In recent years different tetrazolium salts, such as redox sensitive formazan [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide](MTT) (Mosmann, 1983), XTT(Roehm et al., 1991) or MTS (Buttke et al., 1993) have been described. The salts are cleaved to formazan by cellular enzymes, and the concentration of the product formed directly correlates with the number of metabolically active cells in culture. For this study we used a new non-radioactive cell proliferation reagent (4-[3-(-4-Iodophenyl)-2-(4-nitrophenyl0-2H-5-tetrazolio]-1,3-benzene disulphonate] (WST-1) (Tan and Berridge, 2000) for the following reasons: 1. In contrast to MTT, it cleaves to a water-soluble formazan crystal, which can be measured directly. 2. It is more stable in comparison to XTT and MTS 3. It has a wider linear range and shows accelerated color development compared to XTT. 4. The assay itself does not involve any manipulations of the treated cells (no washing, harvesting and

solubilization needed) and hence is easy to conduct and particularly applicable for non-adherent cells like CTLL-2 cells. 5. It is flexible and easy to optimise, the plates can be read and returned several times to the incubator for extended color development. In these experiments, we compared the sensitivity of WST-1 with [^{3}H]TdR-based analyses for evaluation of the proliferation of the CTLL2 cells. Initially, $2x10^4$ cells were treated with different concentrations of IL2 for 24 H followed by WST-1 or [^{3}H]TdR assay based analyses. The cell proliferation analyses showed that both the [^{3}H]TdR and WST-1 assays could detect IL2 concentrations down to 0.19U/mL, although the background error was much higher with the WST-1 assay at 24 h incubation (Figure II, Panels A and B). We then attempted to optimise the WST-1 assay to achieve better sensitivity and our data showed that comparable sensitivity to the thymidine incorporation method could be achieved when $5x10^3$ CTLL-2 cells were incubated for 48 h (Figure II, Panel C). Importantly, a significantly improved dynamic range was achieved after a 72 h incubation (Figure II, Panel D) in comparison to [^{3}H]TdR assay. This was significant in view of the fact that MTT based assays showed 10 fold less sensitivity than tritiated thymidine incorporation assay (Russell and Vindelov, 1998). For all subsequent experiments, we used the WST-1 assay for measuring the CTLL-2 cell response with the following conditions: $5x10^3$ CTLL-2 cells for 72 h with different treatments and incubation with WST1 for 4 h before measurement of color formation (absorption) at 450nm.

3. Evaluation of the proliferative response of CTTL2 cells to the presence of recombinant mIL12 in the growth medium.

CTLL-2 have been shown to require IL2 not only for proliferation but also for survival (Deng and Podack, 1993). The integrity of the CTLL2 cells for all experiments was tested each time through evaluation of their IL2 responsiveness via measurement of their proliferation after a 72 h incubation with different doses of IL2.

Given that the proliferative effect of mIL12 and mIL18 on CTLL2 cells was being assessed, the normal culture doses of IL2 (20Units/mL) would have masked the effects of other cytokines.

Preliminary optimisation experiments were done to determine a submitotic concentration of IL2 that would allow the cells to survive but not proliferate. Cells were incubated with different doses of IL2 (0.19-50 U/mL) and analysed for proliferation at 24, 48 and 72 h (data not shown). *These data suggested that at 2U/mL of IL2, CTLL-2 cells were able to survive for 72 h without proliferating.* From this point on all assays involving mIL12 and mIL18 were done using assay media containing 2U/mL of IL2.

i) CTTL2 cells respond to recombinant mIL12 in the growth medium CTLL-2 cells ($5x10^3$) were incubated with recombinant mIL12 at different doses ranging from 0-12.5 ng/mL for 72 h; this range was chosen on the basis of activity determined by the commercial suppliers (0.5-1 ng/mL). Despite the advantages, a problem with cell line based bioassays is that it is hard to find a cell line which responds to only one cytokine. We overcame that by including the cytokine specific antibody in the cultures. The incubations were performed with or without the neutralising IL12 antibody at 1 μg/mL. Data from a representative experiment is shown (Figure III, Panel A, Graph 1). The proliferation of CTLL-2 cells in response to mIL12 was clearly detectable ($p=0.01$) even at the lowest dose tested (0.5 ng/mL). Please note that we were unable to detect any responses at doses lower than 0.5 (data not shown). The enhancement of proliferation was greatest (7 fold) at 12.5 ng/mL of mIL12. Importantly, this enhancement was completely abrogated in the presence of αmIL12 antibody, indicating the IL12-specific nature of this response. These data indicate that the cell based assay was comparable to the sensitivity of the traditional splenocyte based IFNγ release assay (Figure III, Panel A, Graph 2); we could detect mIL12 upto 10ng/ml and no significant change was detected from 10-20 ng/mL of mIL12. We conclude that CTLL-2 cells proliferate in response to the presence of mIL12 in a cytokine specific manner and that this would provide a reliable and sensitive assay for detection of functional mIL12 in biological samples.

ii) CTTL2 cells do not respond to the presence of recombinant mIL18 in the growth medium. Next, the proliferative response of CTLL-2 cells to mIL18 was evaluated. Given the low percentage of

mIL18 receptors on CTLL-2 cells, we hypothesised that CTLL-2 cells may not proliferate in response to mIL18 in the media. Cells in 2U/mL IL2 were incubated with recombinant mIL18 at different doses ranging from 0-100 ng/mL (on the basis of the activity determined by the supplier, ED50: 20-50ng/mL). The incubation was done in the presence or absence of anti-IL18 antibody at 2µg/mL (Our laboratory, personal communication). At 72 h post treatment, the cells did not show any significant proliferative response to mIL18 ($p>0.05$). Some enhancement of proliferation was observed at the highest dose of 100µg/mL (Figure III, Panel B). While not significant, the marginal response was obvious and was reduced in presence of mIL18 neutralising antibody. This minor response could be attributed to a small percentage of mIL18 receptor expressing cells. In contrast, when we employed the traditional IFNγ release assay we could detect mIL18 at 10ng/mL which increased in a dose dependent manner up to 50ng/mL but the increase was insignificant at 100 ng/mL of mIL18. This suggested that the sensitivity of IFNγ release assay was within the10-50ng/mL range and saturation is reached beyond this point. This correlated with the activity reported by the commercial supplier.

iii) CTLL2 proliferation is further enhanced when treated with both mIL12 and mIL18 in tandem.

As the response of CTLL2 cells to mIL18 was insignificant, experiments were designed to evaluate if the two cytokines in combination would lead to enhanced responsiveness of CTLL-2 cells. *The rationale was that initial incubation with mIL12 would upregulate the mIL18 receptors in CTLL2 cells and this may lead to an enhanced response of the CTLL2 cells to mIL18 as a follow up treatment*. *This could then be developed as an assay to assess functional mIL18 or both cytokines in samples*. A similar study done by Ahn et al (Ahn et al., 1997) using 2D6 cells showed that these cells were responsive to mIL18 only when they were pre-treated with mIL12. The authors also showed that this was due to the increase in mIL18 receptors on these cells after mIL12 treatment (Ahn et al., 1997). In the first experiment, CTLL-2 cells were treated with 0-100 ng/mL mIL12 and 24 h later, mIL18 was added to the cultures at 0-100 ng/mL (Figure III, Panel C, Graph1). There

was significant increase in absorbance at all pre-treatment doses of mIL12 ($p=0.003$); the responses were significantly greater than when either cytokine was used alone. Further, mIL18 could be detected at the lowest levels tested, 1ng/mL In comparison, we were not able to detect levels lower than 10ng/mL using the standard IFNγ release assay employing purified T cells from murine splenocytes (data not shown and Figure II, Panel B, Graph 2). We obtained 10 fold enhancement in sensitivity in comparison to that obtained using the conventional mIL18 bioassay when 10 ng/mL of mIL12 was used with 10-100 ng/mL of mIL18 ($p=0.007$). While significant, this increase was substantially reduced when mIL2 was increased to100 ng/mL. This may be due to toxicity at high doses of mIL12.

In the next experiment we aimed to show that enhancement of proliferation is specific to mIL18 and mIL12. CTLL-2 cells pre-treated with 10 ng/mL of mIL12 were incubated with 10 and 100 ng/mL of mIL18 for 72 h. These concentrations were chosen based on previous experiments; CTLL2 proliferation and survival were best at these concentrations/combination. The incubations were done in the absence or presence of αmIL12 and/or αmIL18 antibodies. Cells treated with both cytokines showed enhanced proliferation compared with either alone (Figure III, Panel C, $p<0.01$). This enhancement was the most significant (~2 fold, $p<0.01$) when 10 ng/mL each of mIL12 and mIL18 were used and was reversed when αmIL12, αmIL18 or αmIL12+αmIL18 antibodies were used, indicating the specificity of the response to individual cytokines. While not significant, the reduction was more pronounced when the two antibodies were used together. There were some inter-assay variations. In the previous experiment there was up to 10 fold enhancement when 10 ng/mL of mIL12 was used with 100 ng/mL of mIL18. We could only achieve an enhancement of 2-fold in this experiment (Figure III, panel B Graphs 1 and 2). However, we always got consistent trends in all experiment. A number of factors could have contributed to this:

1) Batch differences between CTLL2 cells: we often started with a new batch of cells to avoid high passage numbers. 2) Variations in activity of different batches of the recombinant cytokines. 3)

Different batches of FCS: We noted that the IL-2 responsiveness of CTLL-2 cells affected their response to these cytokines; this was supported by the observation that a subpopulation of these cells becomes IL2 independent when in culture for long periods (Giglia et al., 1985). In our preliminary experiments we found that the responses of CTLL-2 cells were not consistent from one experiment to next, hence we tested the cells at an early (8-10 passages after thawing) and late passage (30-40 passages after thawing) for IL-2 responsiveness. Our results clearly indicated that CTLL-2 cells showed best results when used at an earlier passage (8-10); this correlated with their performance in mIL12 and/or mIL18 bioassays (data not shown). As the passage number increased the IL-2 responsiveness of the cell lines decreased and cells at passage 40 could thrive independently of IL2.

4. CTLL-2 based bioassay for detection of functional mIL12 and mIL18 in biological samples.

In these experiments the aim was to assess CTLL-2 based bioassay for detection of functional mIL12 and mI18 in biological samples. For this we used supernatants containing mIL12 or mIL18 from human kidney, 293A cells. To generate these supernatants, we infected these cells with recombinant Adenovirus expressing mIL12 (AdmIL12, A.khatri) or mIL18 (AdmIL18, RIKEN, Japan) at a multiplicity of infection of 10 plaque forming units (pfu)/cell and harvested the cell supernatants at 48 h post infection. The cytokines secreted into the media were detected and measured by mIL12 or mIL18 ELISA. Supernatants from 293 cells infected with AdGFP were used as negative controls.

CTLL2-based bioassay for detection of mIL12 in supernatants: The CTLL-2 cells in 2U/mL of IL2 were incubated with 2-fold serial dilutions of the AdmIL12/293 ([mIL12]:161.75 pg/mL-0.31 pg/mL) or AdGFP/293 supernatants ([mIL12]: 0 pg/mL) with same protein contents for 72h. Cell proliferation measured by WST-1 assay clearly showed that CTLL-2 based bioassay successfully detected mIL12 in supernatants from AdmIL12/293 cells, which wasn't observed when control ADGFP/293 supernatants were used (Figure IV, Panel A, Graph 1, $p<0.0001$). This suggested the

IL12-specificity of the bioassay. Detection was best between 96.5 pg/mL -0.75 pg/mL of mIL12 (as determined by mIL12 ELISA). This sensitivity level was comparable to that observed with the conventional IFNγ release assay (Figure IV, Panel A, Graph 2, $p<0.0001$). While the trends observed were similar in the two methods, the IFNγ release assay was more sensitive at higher concentrations of mIL12 in comparison to CTLL-2 bioassay. Cytokines present in the biological samples are present in a complex mixture of other molecular isoforms, cytokines and factors which may affect the sensitivity and performance of the bioassay (reviewed in (Meager, 2006). It is possible that at lower dilutions, even though the concentrations of mIL12 are higher, the other cytokines and factors affected the CTTL-2 cell responses. Hence it is crucial to analyse a wide range of dilutions to accurately assess the active cytokine.

Overall, CTLL-2 bioassay clearly demonstrated similar levels of mIL12-specificity and sensitivity to that of the IFNγ release assay thus providing a novel and convenient alternative method. Similar results (5-6 fold compared to the controls) were obtained when a murine prostate cancer cell line (ras-myc activated, RM1) (Thompson et al., 1993a) stably transformed to produce secreted mIL12 or mIL18 (RM1GFPmIL12) was used with RM1 cells stably transformed to express GFP (RM1GFP) as controls (data not shown). These data showed the reliability of the CTLL2 responses when mIL12 was obtained from another cell source.

CTLL2-based bioassay for detection of mIL18 in supernatants: CTLL-2 cells in 2U/mL of IL2 were incubated with 0 or 20 ng/mL of recombinant mIL12 for 24 h followed by addition of 2-fold serial dilutions of the AdmIL18/293 supernatants with or without mIL18 neutralising antibody. A biphasic dose response was observed in both CTLL-2 based and IFNγ-release assays (Figure IV panel B). Absorbance readings at 72 h showed that that CTLL-2 based bioassay successfully detected mIL18 in supernatants from AdmIL12/293 cells; this effect was significantly reduced to the base levels in the presence of mIL18 neutralising antibody or in the absence of mIL12 (Figure IV, Panel B, Graph

1, $p<0.0001$). Apart from reaffirming our earlier observations, these data clearly established the specificity of this assay. We could detect mIL18 at all doses (0.04-19.17 ng/mL of mIL18 as determined by ELISA). The sensitivity of the assay was comparable to that observed with the standard IFNγ release assay (38.35-0.04 ng/mL) (Figure IV, Panel B, Graph 2, $p<0.0001$).

For both cytokines, the CTLL-2 responses were greater despite the low concentrations in supernatants (measured by ELISA) in comparison to when commercial cytokines were used (Figure III and IV). Much lower concentrations of the cytokines were detected in biological samples. Both CTLL-2 and IFNγ-release assays showed the same pattern. This may be due to loss of activity in the commercial preparations, as it is common knowledge that once reconstituted, purified cytokines often lose activity in proportion to storage time.

DISCUSSION AND CONCLUSION

We have demonstrated the promise of an IL-2 responsive murine cell line, CTLL-2 for developing a convenient and reliable alternative to the existing bioassays for detection of murine cytokines, IL12 and IL18. The responses of the cells to the cytokines were cytokine specific and reproducible. Importantly the sensitivity of the assay was comparable to that of the conventional IFNγ-release assay. The study was initiated largely because 1. The required cell lines 2D6 (for mIL12 assay) and KG/murine IL18 receptor were not easily accessible and relatively less characterised. 2. We wanted to avoid the use of animals to perform the splenocyte based IFNγ release assay for our studies.

An important prerequisite for a cell line to be suitable for a bioassay is that it is well characterised and easily accessible to laboratories around the world.

CTLL-2 is the best characterised murine T cell line to date. It is the most exhaustively used cell line for many applications: They have been used for the bioassay of IL2 in both human and murine

samples, and for IL15 and indirect conversion assays to detect other growth factors e.g. IL1 (H.Ibelgaufts, ; Meager, 2006). Their culture conditions and freezing protocols have been optimised to improve their integrity and survival (Boise et al., 1996; Weston et al., 1998). CTLL-2 responses to other growth factors e.g. IL1, IL3, IL4, IL5, IL6, IL7, IL10, IL12, IL13, Granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor β (TGF-β) have been investigated (Burton et al., 1994; Weller et al., 1994). Amongst the murine cytokines CTLL-2 responds to murine IL4 apart from mIL2.

The potential of CTLL2 cells for this study was clear because i) our preliminary studies indicated the presence of mIL12 receptors on these cells suggesting that they may respond to the presence of mIL12 in the media and ii) although the mIL18 receptors were minimal on these cells; there was potential to develop the assay further for its detection on the basis of proven synergy between the two cytokines. Upon optimisation, the measurement of cell proliferation based on non-radioactive, WST-1 was found to have enhanced or similar levels of sensitivity compared to the traditional radioactive tritiated thymidine incorporation assay. The WST-1 assay had the additional benefits of simplicity, convenience and versatility. While CTLL-2 responses to mIL12 and/or mIL18 were measured at submitotic doses of IL2 (2U/mL) to minimise masking their effect, in later experiments we found that increasing the IL2 to 5U/mL did not influence the outcome and similar trends were observed (data not shown). Despite the advantages, a major problem with cell line based bioassays is to the difficulty of finding a cell line which only responds to one cytokine. While the response of CTLL-2 cells to various human cytokines has been evaluated, it has not been tested against murine cytokines, with the exception of murine IL4. Inclusion of appropriate neutralising antibodies ascertained the specificity of these responses. Unlike its human counterpart (Schoenhaut et al., 1992; Burton et al., 1994), murine IL12 led to a proliferative response in CTLL-2 cells. That the sensitivity of the assay was comparable to that obtained with the traditional IFNγ-release assay was clearly shown when either recombinant purified cytokine (known concentrations) or cytokine containing

supernatants (non-purified cytokines at unknown concentrations) were used. The proliferative effects were not significant in the presence of IL12 neutralising antibodies and the use of control supernatants substantiated the specificity of the response. When assayed for mIL18, the CTLL-2 response was not significant. Again this was expected given the low percentage (~5%) of receptors on the cell surface. Based on numerous studies showing the synergism between IL12 and IL18 including their murine counterparts (Ahn et al., 1997; Chang et al., 2000; Liebau et al., 2002; Liebau et al., 2004; Li et al., 2005) and the upregulation of IL18 receptors by IL12 as a putative mechanism (Ahn et al., 1997), we designed a bioassay for mIL18 based on pre-treatment of cells with IL12. Our studies clearly reaffirmed the synergistic interaction between the two cytokines. Pre-treatment with mIL12 clearly lead to enhanced sensitivity of CTLL-2 cells to mIL18 in a cytokine specific manner as shown by the significant reduction when neutralising antibodies to either or both cytokines. Typically, the blood levels of many interleukins including IL2, are much lower than 1 pg/ml except during conditions of exaggerated immune response and in certain diseases. In the final experiments (Figure IV), we could detect mIL12v and mIL18 at concentrations as low as 0.75 pg/mL and 40 pg/mL, respectively. This is much lower than the resting levels of the two cytokines in healthy C57/B6 mice sera; mIL12 at 0.2 ng/mL (unpublished results, A.Khatri) and 260 pg/mL (Hoshino et al., 2001).

In general inter-assay variations were observed, but the trends were consistent and significant. A number of factors could explain these variations:

1. Depending on the source and properties of different cell types: It is known that apart from IL2, CTLL-2 cells show a proliferative response to the cytokine IL-15 (proliferative), murine IL4 and anti-proliferative responses to TGF-β1 and TGF-β2 (Weller et al., 1994; Chung et al., 2000). Hence supernatants from cells that produce inhibitory factors such as TGFβ under certain conditions (Thompson et al., 1992; Thompson et al., 1993b) may counteract the proliferative stimulus of these cytokines.

2. Batch variations in CTLL2 cells: Cultured cell lines often lose their responsiveness if cultured for long periods (Mire-Sluis et al., 1995a; Mire-Sluis et al., 1995b). It is known that CTLL-2 cells in continuous culture can vary in IL2 sensitivity and can lose IL2 responsiveness when cultured for long terms (Giglia et al., 1985; Weston et al., 1998). As discussed in the results, we found that IL2 responsiveness of the CTLL-2 cells directly correlated with their response to mIL12 and mIL18. Further, this IL2 responsiveness was gradually reduced with the length of the culture.
3. Cytokines: Cytokine preparations can vary structurally e.g. post translational modifications depending on the source, constituents of the solutions. Commercial cytokines are often prepared in buffered saline solutions containing a carrier protein to stabilise the cytokine or as a bulking agent for lyophilisation. e.g. human serum albumin. Once reconstituted the cytokine preparations can have variable activity especially at temperatures above -20^{o}C. In contrast, cytokines present in the biological samples are present in low concentrations in a complex mixture of other molecular isoforms, cytokines and factors which may affect the sensitivity and performance of the bioassay (reviewed in (Meager, 2006).
4 Mycoplasma contamination and batch variations in fetal calf serum could also lead to variations (Mire-Sluis et al., 1995a; Mire-Sluis et al., 1995b).

Bioassays based on cell culture are inherently variable hence they must be monitored for sensitivity and specificity on an assay to assay basis by including appropriate controls (including the relevant neutralising antibodies) and standards using the homologous cytokine with known concentrations. A careful design of the bioassay with stringent maintenance standards e.g. FCS batch testing, regular mycoplasma testing and inclusion of experimental controls would minimise the inter-assay variations. Short term culturing of CTLL2 cells to avoid higher passage numbers and regular and stringent batch testing would be crucial to the successful performance of these assays. Establishment

of the integrity (IL2 responsiveness) of these cells every time an assay is to be performed would potentially avoid any data discrepancies.

Thus, we have shown that CTLL-2 cell line based bioassay can be further developed to accurately measure the functional titres of murine cytokines mIL12 and mIL18 in biological fluids. Appropriately controlled, this assay has the potential to be more specific, precise and as reproducible as the immunoassays.

Acknowledgements: We acknowledge the Department of Defence, USA and Rebecca L Cooper Foundation, Australia for supporting this work. We also thank Ms Lara Perryman for her help and Mr. Steven Schibeci for his valuable advise during this study.

FIGURE LEGENDS

Figure I: Assessment of mIL12 and mIL18 receptors on CTLL-2 cells: **Panels A and B** show flow cytometry-based analyses of CTLL-2 cells immunostained with anti mIL12 or anti-mIL18 receptor (Panel B) antibodies, respectively. Cells stained with the secondary antibody alone (not shown) or equivalent amounts of the isotype controls served as the controls. **Panel C** shows quantitative assessment of the two receptor types on CTLL-2 cells in a tabular form.

Figure II: Comparison of WST-1- and Thymidine incorporation-based quantitation of CTLL-2 cell proliferation: $2x10^4$ CTLL-2 cells were treated with different concentrations of the cytokine IL2 for 24 h and then either 10 μL of WST1 or 0.5 μci of Tritiated Thymidine were added per well. For the cells with WST-1, the absorbance was measured at 450 nM after 4 h incubation with WST-1. The Tritiated Thymidine incorporation by the cells was measured after 8 h incubation using an automated cell harvester and analysed by the software 'Top Count'. Data are normalised for the zero values. **Panel A** shows the Graphs obtained after 24 h incubation of CTLL-2 cells with IL2 using

the two different methods. **Panel B** shows the absorbance at 450 nm after $5x10^3$ CTLL-2 cells were incubated with IL2 for 24 h. **Panel C** Absorbance at 450 nm after $5x10^3$ CTLL-2 cells were incubated with IL2 for 48 h. **Panel D** Absorbance at 450 nm after $5x10^3$ CTLL-2 cells were incubated with IL2 for 72 h.

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Figure III: Panel A: Response of CTLL2 cells to the cytokines IL2, mIL12 and mIL18: To evaluate the IL12- responsiveness of CTLL-2 cells, cells were maintained in submitotic doses of 2U/mL of IL2 as determined from their IL2 dose response curve. These were then treated with different concentrations of mIL12 with or without anti mIL12 antibodies. ***Graph 1*** in Panel A shows the absorbance measured at 450nM, 72 h post treatment of CTLL-2 cells with mIL12, with and without the neutralising antibodies. ***Graph 2*** in Panel A shows the shows IFNγ released by purified T cells (mouse splenocytes) 72 h after treatment with mIL12.

Panel B: Evaluation of proliferative response of CTLL-2 cells to mIL18: ***Graph 1*** absorbance measured at 72 h when CTLL-2 cells were treated with mIL18, with and without the neutralising antibody. Some proliferative responses could be detected at higher doses of mIL18 but the increase was insignificant. ***Graph 2*** in Panel B shows IFNγ released by purified T cells (mouse splenocytes) 72 h after treatment with mIL18.

Panel C: ***Graph 1*** shows the CTLL2 proliferative responses at 72 h post treatment, when mIL12 and mIL18 were given in combination at different ratios in tandem. CTLL-2 cells maintained in 2U/mL of IL2 were treated with mIL12 at different doses for 24 h and then treated with different doses of mIL18 for further 48 h. The absorbance was measured at 450nm. ***Graph 2*** demonstrates the cytokine-specificity of the response by evaluating the proliferative responses of CTLL2 cells in the presence of antibodies to mIL12 and/or mIL18. * denotes statistical significance ($p<0.01$).

Figure IV: <u>Assessment of CTLL2- cell based bioassay for detection of functional mIL12 and mIL18 in laboratory samples</u>: CTLL-2 cells maintained in 2U/mL of IL2 were incubated with supernatants obtained from 293 cells infected with AdmIL12 or AdmIL18 (moi of 10 pfu/cell; 48H) for 72 h. The concentrations of cytokines in the supernatants were determined by ELISA based immunoassay. The protein concentration of the supernatants was determined using the BCA-based protein estimation kit. Supernatants from 293 cells infected with AdGFP were used at the equivalent protein concentrations as controls. **<u>Panel A</u>**: ***Graph 1*** shows proliferation of CTLL2 cells maintained in 2U/mL of IL2 after 72 h incubation in supernatants from AdmIL12 or AdGFP infected 293 cells. ***Graph 2*** shows IFNγ released by purified T cells (mouse splenocytes) 72 h after the same treatment. **<u>Panel B:</u>** ***Graph 1*** shows the proliferation of CTLL2 cells pre-treated with 0 or 10 ng/mL of mIL12 in response to supernatants from AdmIL18 infected 293 cells at 72 h. ***Graph 2*** shows IFNγ released by purified T cells (mouse splenocytes) after 72 h incubation with AdmIL18/293 supernatants with AdGFP/293 supernatants as controls.

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Figure(s)

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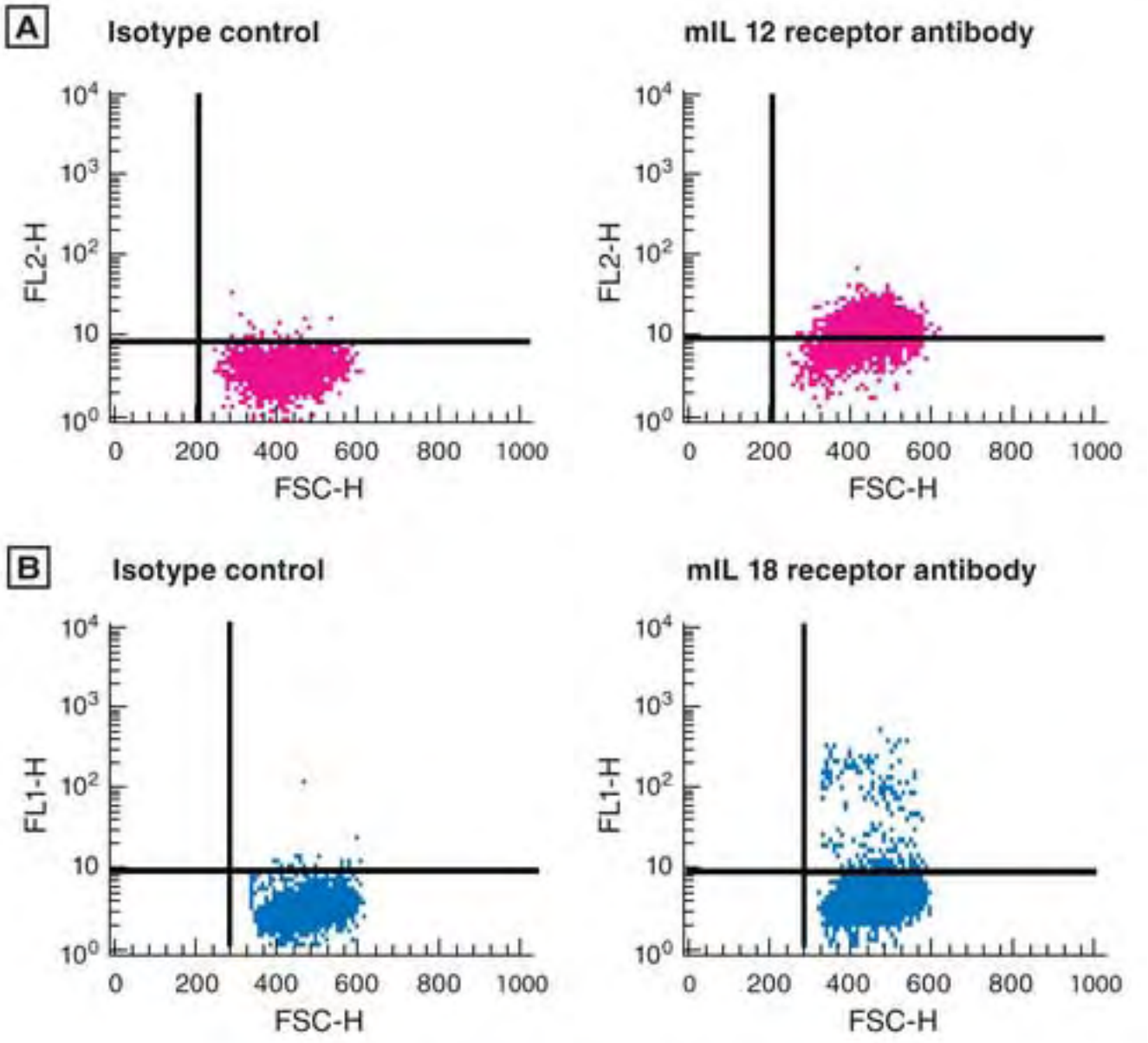

Type of receptor	% of expressing CTLL2 cells
mIL12	65-70
mIL18	5-6

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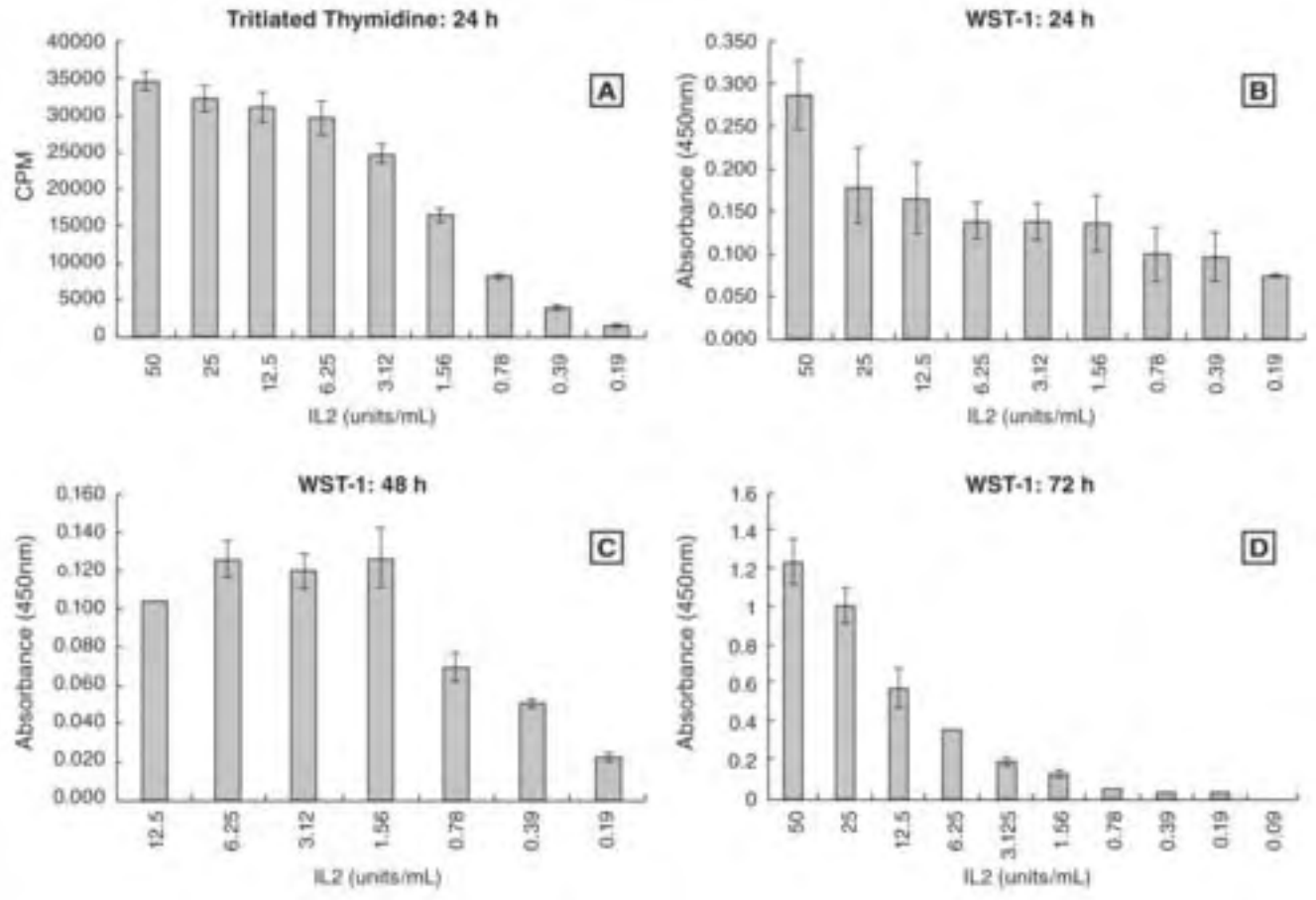

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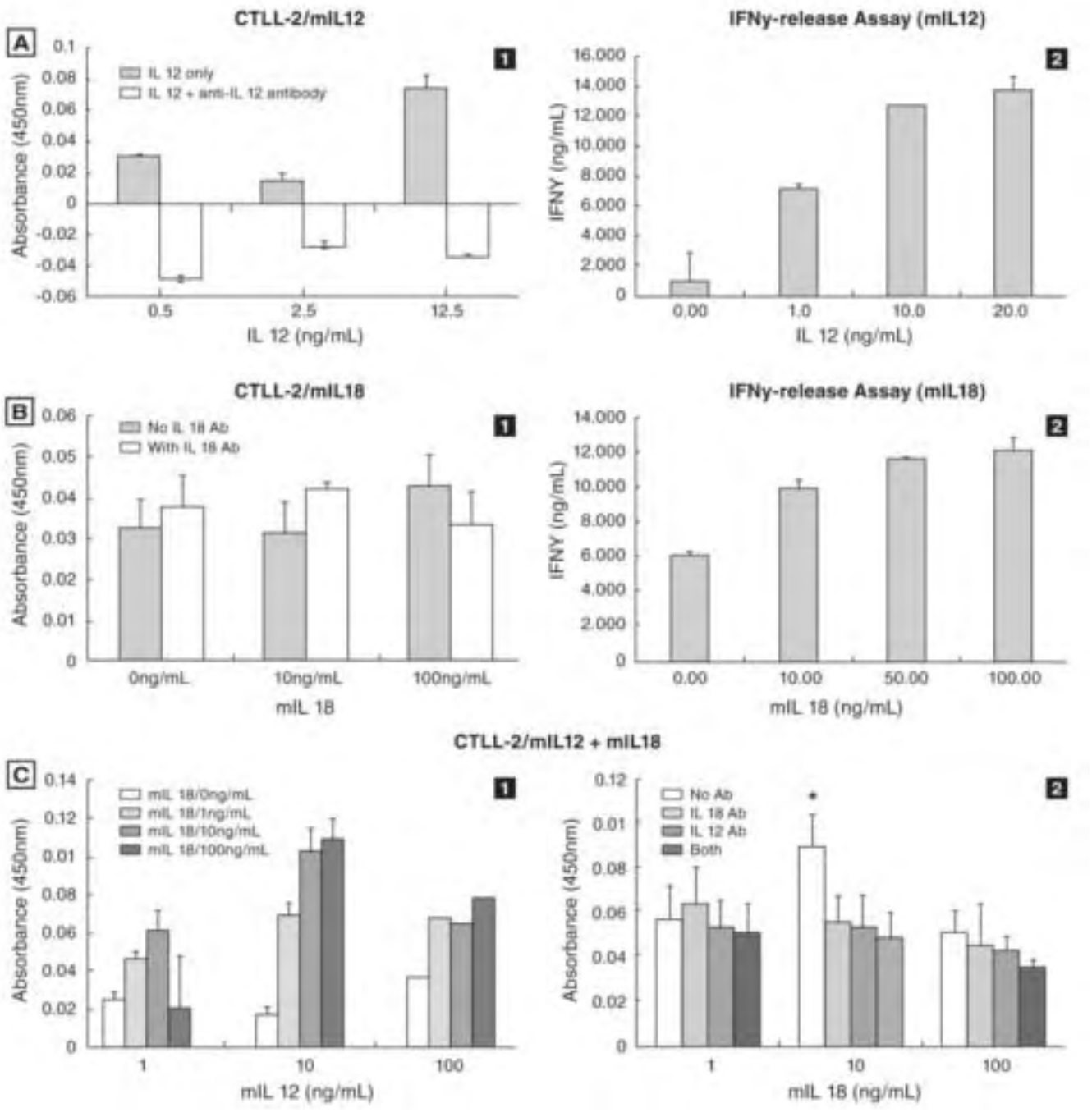

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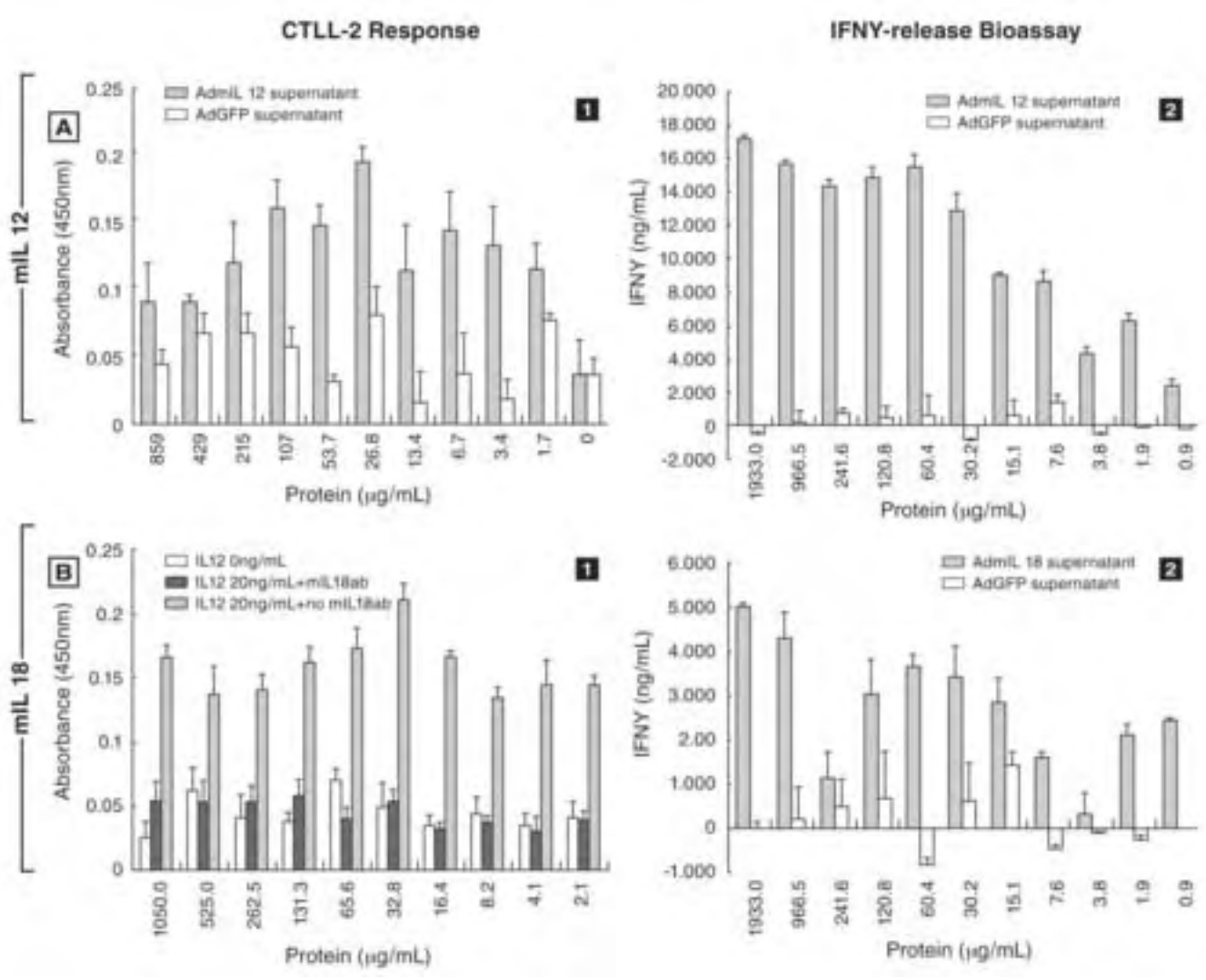

Broadening of transgenic adenocarcinoma of the mouse prostate (TRAMP) model to represent late stage androgen independent cancer

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Introduction:

Prostate cancer (PC) is the most common visceral cancer and the second leading cause of cancer related death in men in Western countries (1,2). In Australia, 2700 men die of prostate cancer every year and the overall lifetime risk of developing PC is now 1 in 6 men (3). The most significant development has been the advent of prostate specific antigen (PSA) testing, which has led to a shift from diagnosis at advanced-stage towards early-stage disease. Treatment for local disease is conventionally prostatectomy or radiation, however, the cancer recurs in some 40% of patients and eventually progresses from androgen dependent to a drug-resistant androgen independent (AI) hormone refractory (HRPC) phenotype. The only option for late stage metastatic HRPC is still palliation although recently, in a couple of landmark trials, docetaxel was found to be effective in 40% of patients, however there were unwanted side effects(4,5). Clearly, a better understanding of the underlying mechanisms of disease progression and new therapies are needed, a process that would be greatly facilitated by the availability of well-characterised and clinically relevant animal models that closely represent the biological progression and logistical challenges of the human disease. Although, xenogenic murine models of PC have proven useful in various pilot and proof-of-principle studies, they do not recapitulate the significant biological complexities of the human disease**.** A transgenic mouse model of PC that encompasses all stages of disease would be ideal**.** In that regard, the transgenic mouse adenocarcinoma of the prostate (TRAMP) model, first described in 1995, is the best characterised. It closely mimics PC as it occurs in men (6) and hence is an attractive model to evaluate preventative and therapeutic strategies including immunotherapy. TRAMP males spontaneously develop adenocarcinoma of the prostate; the general time frame of the disease progression from local prostate confined hyperplasia (PIN) to metastases (lymph node

and lungs) ranges from 10-30 weeks. After castration at 12 weeks of age, poorly differentiated, HRPC develops in TRAMP mice by 24 weeks (7). While clinically relevant, the logistical and technical challenges due to inherent variability of disease incidence and long latent periods and hence the long term for experimentation leads to difficulty with conducting synchronous experimentation in a timely manner. This was partially addressed by establishment of tumorigenic cell lines (TRAMP C1 and C2) from TRAMP tumours; apart from TRAMP mice these could also be transplanted into immunocompetent syngeneic C57BL/6 mice (8). The androgen sensitivity of these lines limits the scope of investigative studies. Further, studies done in our laboratory (9) have shown that it takes up to 4-6 weeks to reach 5x5mm ($65mm^3$) and the growth rate is very slow (80-95 days to reach 15x15mm ($1767.1mm^3$) for these cell lines with TRAMP-C1 being marginally faster growing than TRAMP C2 (10). In this study, we have expanded the TRAMP model to include cell lines that represent late stage, androgen independent (AI) disease with shorter latency to facilitate synchronous experimentation whilst minimizing time and financial requirements. The development and characterization of androgen independent cell lines derived from TRAMP C1 and C2 cells is described.

Materials and Methods:

1. Cell Culture:

TRAMP parental cell lines TRAMP C1 (TC1) and TRAMP C2 (TC2) (from Dr. Norman Greenberg, Fred Hutchinson Cancer Research Centre, Seattle, WA) were cultured in the presence of 10^{-8} M Dihydrotestosterone (DHT) as described (8). Androgen independent (AI) cell lines derived from parental TC1 and TC2 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% charcoal dextran stripped foetal calf serum (FCS) [Product notes-Sigma Aldrich, MO, USA; (11)]. LNCaP.FGC and PC-3 cells were cultured in RPMI 1640 supplemented with 10% FCS (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2-humidified incubator.

Primary cultures: To generate primary cultures, PC tumours (5x5mm) were mechanically chopped into <1 mm size fragments using a scalpel blade then enzymatically digested using collagenase (1:10 in serum free medium; Sigma Aldrich, MO, USA) for 1 h at 37^0C before plating. Cultures were monitored for growth every 2-3 days until continuous cultures were established (4-6 weeks).

2. Doubling Time *in vitro*

Doubling times of the cell lines were determined by plating 20,000 cells/well in six-well plates in triplicate. Growth was measured by counting viable cells via trypan blue exclusion of dead cells every 24 h for six days. The growth rate was analysed from the log phase of cell-growth and calculated using the formula:

Incubation time (h) X $\log10^2$ / Log10 [Final cell number] – log 10[Initial cell number]

3. Clonogenic Assay

Anchorage independent growth of different cell lines was evaluated using the soft agar colony count assay in two different experiments. Cells were seeded ($5x10^4$cells/well) in triplicate in a six well plate. Colonies (≥20 cells) were counted after ~ 14 doubling times for sublines and parental cell lines; human PC-3 cells were used as positive controls.

4. Matrigel Invasion Assay

The invasive ability of the cell lines was determined using a transwell chamber assay as described (12). Briefly, matrigel (BD Biosciences, NJ, USA) was thawed overnight on ice and diluted 1:5 in ice cold serum free medium (tips and tubes were chilled at -20^0 C prior to use). Matrigel (100μL-diluted 1:5) coated transwell filters were, dried overnight and then reconstituted in 100 μL serum-free medium with continuous shaking at 37^0 C for 90 min. Cells ($5x10^3$) in 200 μL medium (2% FCS for parental cells or androgen stripped FCS for derivative cell lines) were added to each transwell and medium (700 μL) was added to the outer well to prevent dehydration. Cells were

incubated at 37^0 C in 5% CO_2 for three doubling times, and then stained with filtered 0.5% crystal violet in PBS for 10 min at room temperature (RT). Excess dye was washed away with tap water and the number of crystal violet stained cells that invaded through the matrigel was scored in five fields by light microscopy.

5. RNA Isolation and cDNA synthesis

Total RNA from each cell line was extracted using tri-reagent (Molecular Research Centre, Cincinnati, OH, USA) according to the supplier's instructions. The purified RNA was quantified by measuring absorbance at 260nm using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA, Australia). Any contaminating DNA in RNA samples was removed using a DNAse kit (Fermentas, Hanover, MD, USA). Next, the single stranded cDNA was synthesized from 1µg of total RNA and reverse transcribed using a Superscript III RT kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, after adding Superscript III reverse transcriptase, samples were incubated at 48^0 C for 1 h followed by enzyme inactivation at 70°C for 15 mins.

6. Quantitative PCR

Expression of androgen receptor (AR) mRNA was evaluated by quantitative real time RT-PCR using a SYBR green qPCR KIT (Invitrogen, Carlsbad, CA, USA) with optimised concentrations of AR specific primers. In general, reactions were hot started at 95^0 C for 7 mins followed by 35 cycles of 94^0 C for 30 sec, 63^0 C for 30 sec and 72^0 C for 30 sec. After amplification, melt curves were analysed from 60^0 C to 99^0 C to check PCR specificity. Amplicon specificity and reaction specificity were further confirmed by electrophoresis using a 2% agarose gel. Fluorescence in each cycle was measured using the Rotor Gene system (Corbett Research, NSW, Sydney, Aus), and the threshold cycle number (Ct) at which the PCR amplification enters log phase was determined for each gene. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control to normalise samples. Standard curves were generated from 2-fold serial dilutions of TRAMP C1 parental cell line cDNA for PCR each time to allow calculation of the concentrations for AR and the internal control GAPDH. All samples were evaluated in duplicate and mean and standard deviation determined.

The following primers were used:

AR forward: [5'-TGCTGCTCTTCAGCATTATTCCAGT-3'] (13)

AR reverse: [5'-CCAGAAAGGATCTTGGGCACTTGC-3']

GAPDH forward: [5'-CCCATTGTGCTGTAGCCGTA-3']

GAPDH reverse: [5'-AAGGGCTCATGACCACAGTC-3']

7. Protein Expression Studies:

a) Immunoblotting was used to assess AR protein expression. Protein samples were prepared by lysing cells in M-PER mammalian protein extraction reagent (Pierce, Rockford, USA); cell-debris was removed by centrifugation (16,000g, 10 minutes, 4^0C).

The protein concentration of the supernatants was determined using BCA protein assay kit (Pierce, Rockford, USA). Equal amounts of protein (50 μg) from all samples were denatured in sample buffer and subjected to SDS-PAGE analysis followed by transfer to nitrocellulose membranes using a semi-dry blotting method. Blots were then probed with anti-AR (Santa Cruz, CA, USA; dilution 1:200) or -ß-actin (internal control to assess the integrity of the protein samples) antibodies (Sigma Aldrich, MO, USA; dilution 1:30,000), followed by incubation with the appropriate Horseradish Peroxidase (HRP) conjugated secondary antibody. Proteins on the blot were visualised using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, USA) and Kodak X-Ray films. Lysates from AR positive human, LNCaP.FGC cells and AR negative PZHPV-7 cells were used as controls.

b) Immunohistochemical Analyses were done to evaluate the cell lines (cultured or tumour sections) for expression of Cytokeratin, AR and E-Cadherin. Cells ($1x10^5$/well) were seeded in 4 (Lab Tek II, Rochester, NY, USA) or 8 chamber slides (BD Biosciences, NJ, USA) followed by 2 min fixation in cold acetone the day after. Endogenous peroxidase activity in the cells was blocked by quenching with hydrogen peroxide (0.03%). Next, to avoid non-specific binding, cells were blocked sequentially in avidin, biotin and 2% IgG free BSA for 10 minutes each. Cells were stained by incubation with anti-pancytokeratin (Dako, Glostrup, Denmark; dilution 1:400), anti-AR (Santa Cruz, CA, USA ; dilution 1:50) or anti-E-Cadherin (BD Biosciences, NJ, USA; dilution 1:5000) antibodies. Isotype controls, rabbit IgG fraction (Dako, Glostrup, Denmark) for Pan cytokeratin and AR and mouse IgG2a fraction (Dako, Glostrup, Denmark) for E-cadherin were used at the same concentration as the primary antibodies for 1 hour at RT. Expression of the metastatasis suppressor gene, KAI-1 (13), AR and cytokeratin was determined by immunostaining the corresponding frozen tumour sections (5 micron) following the same protocol. Biotynlated anti rabbit antibody (Vector laboratories, Burlingame, CA, USA) was used as secondary antibody (diluted 1:200). Specific intracellular immunoreactivity was detected by incubation with avidin-biotin/horseradish peroxidase complex (Vector Laboratories, CA, USA) for 30 min at RT followed by colour development in diaminobenzidine (DAB) chromogen (Dako, Glostrup, Denmark) for 5 mins. The cells were lightly counterstained in hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene, and mounted in Eukitt for analyses by light microscopy. Scoring was done in 10 fields at x10 magnification and % of positively stained cells was determined.

9. Growth Rate *in vivo:*

Cell lines were assessed for growth *in vivo* in castrated and sham-castarated C57BL/6 mice. Mice were surgically castrated (Bilateral) two weeks prior to tumour implantation by standard surgical techniques. Sham castrated mice were used as controls. Cells ($5x10^6$ in 100µl PBS) were injected sub-cutaneously (sc) on the upper right hand flank of castrated and sham castrated mice (n=5/cell line/group). For TRAMP C2 and derivative cell lines, cells were mixed in a 1:1 ratio with matrigel (100µl) (9). The mice were monitored and the tumours were measured twice a week. Tumour volume was calculated using the formula $V= (\Pi/6(d_1 X d_2)^{3/2}$, (14) with d_1 and d_2 being two perpendicular diameters. Mice were sacrificed when the tumour diameter reached 15x15mm or when mice showed signs of distress or loss of condition or weight.

All mice were bred and maintained at the Biological Resources Centre (BRC), University of New South Wales (UNSW, Sydney, Australia). These studies were performed in accordance with the Animal Care and Ethics Committee guidelines of the UNSW and Prince of Wales Hospital, Sydney, Australia.

RESULTS:

1. Derivation of Androgen independent TRAMP cell lines: The androgen independent cell lines were generated from parental TC1 and TC2 cell lines using *in vitro* and *in vivo* approaches (**Figure1**).

In vitro method: This involved hormone deprivation of TRAMP C1 and TRAMP C2 cell lines over a period of 8-10 weeks. Parental TC1 and TC2 cell lines were grown in DHT (androgen) deprived media with 5% charcoal stripped FCS (to remove residual androgen) (7). Less than 0.001% TC1 and TC2 cells survived 8 days and 10 days post-treatment, respectively. Cells were grown in DHT-free medium until surviving cells grew to confluence and from this point on these cells were cultured in androgen-free medium. Parental cell lines grown in media with FCS served as controls. We generated five AI sublines (2 from TRAMPC-1 and 3 from TRAMPC-2 (Table I) through *in vitro* methods.

In vivo method: Androgen-sensitive TRAMP C1 parental cells were grown in female TRAMP mice (n=18) (androgen levels in female mice are almost negligible in comparison to males, being mainly from ovaries and adrenal glands (15)). Tumor growth occurred in only 1 of 18 female mice, 6 months after implantation. Tumor chips from this tumor were implanted sc into 6 female mice 5 of which developed tumors (F1 generation). Primary cultures were generated from explants obtained from these mice (TC1-F1 - Figure 1 and Table I).

Table I- Derivation of androgen independent sublines from TRAMP parentals cell lines.

Parental Cell Lines	***TRAMP C1***			***TRAMP C2***		
Method of Derivation	*In vivo*[1]	*In vitro*[2]		*In vitro*[2]		
Derivative Sublines	**TC1-F1**	**TC1-T4**	**TC1-T5**	**TC2-T3**	**TC2-T4**	**TC2-T5**

[1] The cell line was derived from TRAMP C1 cells grown in female mice.

[2] The cell lines were derived via androgen deprivation in the culture media *in vitro*.

2. Evaluation of growth rates *in vitro*: Initially the *doublig times of all* cell lines were determined by the trypan blue exclusion method. All derivative cell lines (TC1-T5, TC1-F1 and TC2-T5) were significantly faster growing compared with parental TC1 and TC2 cells (Table II). TC1-T5 was the fastest growing (doubling time, 17.5 h) followed by TC2-T5 (19.5h) and then TC1-F1 (21.6 h) compared with parental TC1 (24.8 h) and TC2 cells (24.2 h) (Table-II). None of the cell lines including the parental cells displayed contact-inhibition.

Table II- Doubling times of cell lines *in vitro*:

Cell Line	Doubling Time (Hr)
TC1[1]	24.8
TC1-T5	17.5
TC1-F1	21.6
TC2[1]	24.2
TC2-T5	19.5

[1] Parental cell lines: TC1: TRAMP C1 and TC2: TRAMP C2

3. Evaluation of the epithelial status of AI sublines *in vitro*: Once established, the epithelial origin of AI sublines was determined by staining for cytokeratin (epithelial cell marker) by immunohistochemical analysis of cultured cells and frozen tumour sections (**Figure 2- Panel B and Table III)**. Over 90% of both parental cells and their sublines showed clear positive membrane and cytoplasmic staining confirming their epithelial origin. Human PC cells, LNCaP.FGC (positive

control) showed strong staining while the negative control, human fibroblast MRC-5cells were negative (data not shown).

4. Evaluation of the clonal growth of the derived cell lines *in vitro*: The capacity of a cell line for anchorage independent growth is indicative of its tumorigenicity. We assessed this for the sublines by evaluating their capacity to form colonies in soft agar. The sublines derived from TC1 and TC2 formed significantly higher numbers of colonies (TC1-T5, 78; TC1-F1,47; TC2-T5,69 colonies) compared to the parental cells (TC1, 17; TC2 5 colonies) (**Figure 3, Panel A**). Anchorage independent colony forming ability was enhanced by up to 5-fold in TC1-T5, 3 fold in TC1-F1 cells, and up to 13 fold in TC2-T5 cells ($P<0.001$)compared with the parental lines. These data clearly indicate the potential of the sublines for increased tumorigenicity and invasiveness. Anchorage-independent growth in descending order was: TC1-T5 >TC2-T5>TC1-F1>TC1>TC2.

5. Evaluation of invasive abilities *in vitro*: Invasiveness of cancer directly correlates with advanced stage cancer, hence, we assayed the sublines for ability to invade matrigel over a period of three doubling times for each cell line. There were minimal numbers of invading cells in the parental TC1 (5 cells) and TC2 (0 cells) cell lines (**Figure 3, Panel B**). In contrast, AI sublines showed increased invasive capability: TC1-F1 (157 cells), TC1-T5 (414 cells) and TC2-T5 (483 cells) ($P<0.001$).. The lack of invasion by TC2 cells correlated with their slow growth *in vivo*, mixing the cells with matrigel significantly enhanced their ability to form tumors with enhanced growth rates in mice (9). In contrast, TC1 cells grew faster *in vivo* and did not show any significant change in their growth pattern with or without matrigel. These results indicate that the invasive abilities of the derivative cell lines are substantially greater than the parental cells. Cell lines in descending order of invasiveness were: TC2-T5>TC1-T5> TC1-F1>TC1>TC2. This correlated with their ability for adherence independent growth *in vitro* (see above).

6. Evaluation of expression of biomarkers of advanced stage PC in TRAMP sublines: The cell lines derived from TC1 and TC2 were assessed for expression of biomarkers of late stage disease:

E-Cadherin, a transmembrane glycoprotein, is generally lost in the advanced stages of cancer (16-19). The TC1 sublines showed loss of E-Cadherin expression with only 47% and 40% of the cell population expressing in TC1-T5 and TC1-F1, respectively (**Figure 4- Panel A and Table III**), compared with 70% in TC1 cells. Similarly, E-cadhering expression was reduced TC2-T5 (41% positive cells) compared to 69% in parental TC2 cells (**Figure 4- Panel A and Table III**). Further, the intensity of staining was reduced in the sublines compared with the parental cells **(Figure 4, Panel A)**. Cell lines in descending order of E-cadherin expression were: TC1-F1=TC2-T5>TC1-T5>TC2=TC1.

KAI-1 , a metastasis suppressor gene, has been implicated in the metastatic disease progression in a number of cancers including human PC (20,21). Clinical studies have shown that KAI 1 protein

expression is downregulated in >70% primary prostate cancer and in 100% of lymph node metastases. Further, a study done in patients who died of metastatic disease showed a high incidence (70%) of loss of heterozygosity at the KAI1 locus (22). When introduced into rat metastatic prostatic cancer cells, KAI-1 significantly suppressed metastasis without affecting the tumor growth rate. KAI-1 expression is high in human normal prostate and benign prostatic hyperplasia but is dramatically lower in cancer cell lines derived from metastatic prostate tumors Immunohistochemical staining for KAI-1 demonstrated a significant downregulation in the % of KAI-1 expressing cells in derivative sublines (TC1-F1-4%, TC1-T5-21%, and TC2-T5-6%) in comparison to the parentals (TC1-37% and TC2-33%) (**Fig 4- Panel B and Table III**). While TC1-F1 and TC2-T5 showed minimal staining, TC1-T5 cells contained a heterogeneous population of KAI-1 positive cells, although the intensity of staining in the positive cells was reduced in comparison to that observed in the parental cells **(Figure 4, panel B)**. Cell lines in descending order of KAI-1 expression were: TC1-F1≥ TC2-T5>TC1-T5 >TC1≥TC2.

7. Evaluation of the AR expression: The AR is a ligand-activated transcription factor that mediates the effects of the male sex hormones, testosterone and dihydrotestosterone. Pathological alterations in AR structure and function result in a number of clinical disorders, including androgen-insensitivity, which leads to disruption of male development, spinal bulbar muscular atrophy and PC (23). Thus, The AR status of the sublines was considered to be a key factor in determining their androgen independence. We compared the extent of AR expression at mRNA and protein levels in the sublines to that in the parental cells.

A.) ***Determination of AR mRNA expression:*** AR mRNA was quantified in different cell lines by real time quantitative PCR by comparing the ratio of AR-mRNA to the house keeping gene GAPDH-mRNA (**Figure 5, Panel A**). There was significant downregulation of AR in all three sublines compared with their respective parental cells. TC-F1 and TC2-T5 showed greater down regulation than TC1-T5 in AR mRNA levels (TC1=TC2>TC1T5>TC2-T5>TC1-F1).

B.) ***Evaluation of AR protein by immunoblotting***: We then evaluated the AR protein expression in parental and derived sublines by western blotting analysis. TC1 and TC2 parental cell lines and LNCaP.FGC cells (positive control) were strongly positive for AR (**Figure 5, Panel B**). The subline TC1-T5 showed the presence of AR while no AR bands were detected for TC1-F1 suggesting its complete absence. A faint AR band could be detected in TC2-T5 cells indicative of significant downregulation of AR. Importantly, these data strongly correlated with the real time PCR data. AR protein levels were: TC1=TC2=TC1T5>TC2-T5>TC1-F1

C.) ***Immunhistochemical evaluation of AR***: For qualitative and quantitative assessment of the AR expression, immunohistochemical analysis of different cell lines (cultured cells and corresponding frozen tumour sections) was performed. There was significant down regulation of the percentage of

AR expressing cells in TC1-F1 (11% vs TC1-93%) and TC2-T5 (8% vs TC2-94%) in culture **(Figure5, Panel C and Table III)** or in tumors grown in mice (TC1-F1-9% vs TC1-83% and TC2-T5-10% vs TC2-81%) **(Figure5, Panel D and Table III)**. However, TC1-T5 (51% vs TC1-93%) showed heterogeneous expression suggesting a mixed population of cells; similar results were obtained when tumor sections were stained (e.g. TC1-T5-50% vs TC1-83%). The parental cell lines showed strong staining for AR in culture, however, the staining pattern was more heterogeneous when the corresponding tumour sections were analysed. Overall, the intensity of staining was significantly stronger in parental cells than that observed in the sublines. This heterogeneity could be due to the presence of other cell types in the tumour/sections unlike the cultured cells. The % of AR staining in cell lines was: TC1=TC2> TC1-T5>TC2-T5=TC1-F1

Table III- Summary of quantitative immunohistochemicval evaluation of different markers:

CELL LINES	**Cytokeratin**		**E-Cadherin**	**Kai-1**	**Androgen receptor**	
	% positive cells[1] ± SEM[2]					
	In vitro[3]	*In vivo4*[3]	*In vitro*	*In vivo*	*In vitro*	*In vivo*
TC1	93 (±3)	95 (±1)	70 (±7)	37 (±3)	93 (±3)	83 (±1)
TC1-F1	90 (±1)	93 (±1)	40 (±4)	4 (±3)	11 (±4)	9 (±4)
TC1-T5	93 (±1)	93 (±1)	47 (±7)	21 (±3)	51 (±11)	50 (±2)
TC2	92 (±2)	92 (±2)	69 (±9)	33 (±3)	94 (±2)	81 (±2)
TC2-T5	92 (±2)	90 (±1)	41 (±4)	6 (±3)	8 (±3)	10 (±4)

[1]Cells were counted from 10 different fields and the level of immunoreactivity was graded according to the percentage of positively stained cells.

[2] SEM= Standard error of mean

[3] *In vitro*: cells in culture were immunostained on chamber slides

[4] *In vivo*: Frozen sections from corresponding tumours grown sc in mice were immunostained.

Evaluation of the growth characteristics *in vivo:*

The *in vitro* data clearly indicated that the derivative AI sublines are faster growing than parental cells with increased clonogenicity and invasiveness.

a) Growth rates in vivo: To determine their relative growth rates *in vivo*, we evaluated the sc growth of the sublines in C57BL/6 male mice **(Figure 6, Panel A).** The sublines grew significantly faster than the parental cells (P<.005): TC1-T5 and TC1-F1 tumors grew from ~5x5mm to ~15x15mm in <4 weeks and 5 weeks, respectively in comparison to 9 wks with TC1 cells. **(Figure 6, panel A1 and A3)**. Similarly, the tumour growth was nearly twice as fast in TC2-T5 (5 weeks to 15x15mm tumour size, Panel A3) compared to that of TC2 cells (9 weeks) **(Figure 6, panel A2 and A3)**. The latency of the sublines (time to reach palpable size (~5x5mm) tumours was notably reduced; 2 and 3 weeks for TC1-T5 and TC1-F1/TC2-T5 tumours, respectively, compared to ~3.5 and 4.5 weeks for TC1 and TC2.

b) *Androgen independence in vivo:* To assess the AI phenotype *in vivo* the sublines were grown in castrated and sham castrated mice. The derivative sublines produced tumours at similar rates in both castrated and sham castrated mice clearly establishing their AI phenotype **(Figure 6, panel B)**. While the parental cells grew considerably slower than the sublines, the growth rates were not significantly different between castrated and sham castrated mice. Nevertheless, the TRAMP C1 tumour growth was marginally slower in castrated mice (time taken for tumors to grow from 5x5mm to 15x15mm: sham castrated-4-9 weeks vs. castrated- 5-10 weeks). A similar effect, however, to a lesser extent, was seen in TC2 cells (Sham castrated-5-10 weeks vs. castrated 6-11 weeks. Since our data and other studies have clearly shown that the parental, TC1 and TC2 cells contain a heterogenous mixture of AS and AI cells, it is possible that the more aggressive AI population is more tumorigenic and takes over with time thus explaining the insignificant variation in growth rates between castrated and sham castrated mice observed in our study.

DISCUSSION:

The development of novel therapies for PC relies heavily on clinically relevant preclinical models of cancer. In this study, we aimed to broaden the transgenic mouse model of spontaneous PC, TRAMP, by developing AI cell lines that could be used in conjunction with TRAMP mice or syngeneically transplanted in C57Bl/6 mice for preclinical evaluation of therapy for late stage PC. The TRAMP model while clinically relevant poses the following problems 1. Lack of synchronicity: the inherent variability in timing of tumor incidence and PC growth in mice makes it difficult to design and evaluate preclinical data with accuracy 2. Long latent period of tumour incidence/growth (spontaneously or when using TC1 and TC2 cell lines): draws on resources such as time and money. In order to generate reliable and reproducible data exhaustive experimentation involving multiple experiments is needed; this poses great constraints in terms of time, when using

the transgenic TRAMP model. Generation of syngeneic transplantable cell lines representing different stages, especially late stage PC would address such issues, e.g for conducting pilot studies of greater relevance prior to final testing of a therapy (including immunotherapy) in the transgenic model. The use of TRAMP derived AS TC1 and TC2 cell liens has partially solved these problems but only represented early stage disease.

The normal prostate comprises different cell types; AI basal epithelial stem cells, androgen-dependent luminal secretory cells and androgen-sensitive transitional amplifying cells (24). The ability of these AI basal cells to grow in the absence of androgens is critical in the progression of PC to HRPC. Studies conducted in human xenografts (25) and in TRAMP mice (7) showed that PCs are generally heterogenous and show varying degrees of sensitivity to androgens. This has suggested that clonal selection under androgen deprivation may be responsible for the development of the AI phenotype of PC. Studies in TRAMP mice have clearly established that androgen ablation may be the selective pressure needed to trigger the molecular events leading to metastatic, androgen independent phenotype. Hence we hypothesised that selective pressure of androgen deprivation may be a more relevant approach towards generating AI sublines. Based on this rationale, we have developed cell lines representing different stages of transformation and progression toward AI from AS TRAMP C1 and C2 (7) cells by depleting dihydrotestosterone *in vitro* and *in vivo.* The sublines represent < 0.001 and 0.0001% of the cell populations that survived androgen deprivation, from TC2 and TC1 cell lines, respectively.

The epithelial phenotype of the new sublines was reaffirmed as shown by the positive staining for cytokeratin, a common biomarker of epithelial cells (26). The pan-cytokeratin antibody used for this analysis identifies most of the cytokeratins including cytokeratin 18 and 19, which are specific to malignant cancer cells including PC (27). These sublines clearly demonstrated an enhanced ability for anchorage independent growth and matrigel invasion *in vitro* compared to the parental cells. It was interesting that despite the inability of parentals cell lines to form substantial number of colonies in soft agar, they were tumorigenic *in vivo (our data and* (6). Given that the aim of this study was to establish AI sublines with potential for invasion and metastases, we phenotyped these cells with respect to biomarkers for these functions: our *in vitro* data clearly established that these AI sublines represent different late stage PC phenotypes based on variable AR, E-Cadherin and KAI-1 expression.

We chose to investigate **E-Cadherin** status of these cells as a biological functional relationship between loss of E-cadherin expression and acquisition of invasive behaviour by cancer cells has been reported by several groups (16-19). E-Cadherin is a marker of epithelial cells and mediates adhesion (via catenins); its loss has been associated with increased cell motility and hence invasiveness and poor prognosis. Further, a restoration of the epithelial phenotype and reduction in

invasiveness was reported when E-cadherin was restored via E-Cadherin expressing DNA transfection (28). Consequently, E-Cadherin has been classified as a tumour suppressor gene, the loss of which is important in the cascade of events that lead to metastases leading to poor clinical outcomes in epithelial-derived malignancies (29) (30). In comparison with the parental cells, which showed strong expression both in terms of cell % and intensity of staining, the levels of E-Cadherin expression in the sublines were generally lower, however, there were variations amongst the sublines. The sublines TC1-F1 and TC1-T5 showed a predominance of E-cadherin negative cells with positive cells showing lower levels. In comparison, the subline TC2-T5 had a relatively greater population (10% more) expressing E-Cadherin at low levels with a few expressing at the same level as the parental cells. The mixed population of cells may correspond to different stages of differentiation as shown in hepatocellular carcinomas (31,32) where variability in E-Cadherin expression was related to different grades and stages of differentiation of carcinoma cells

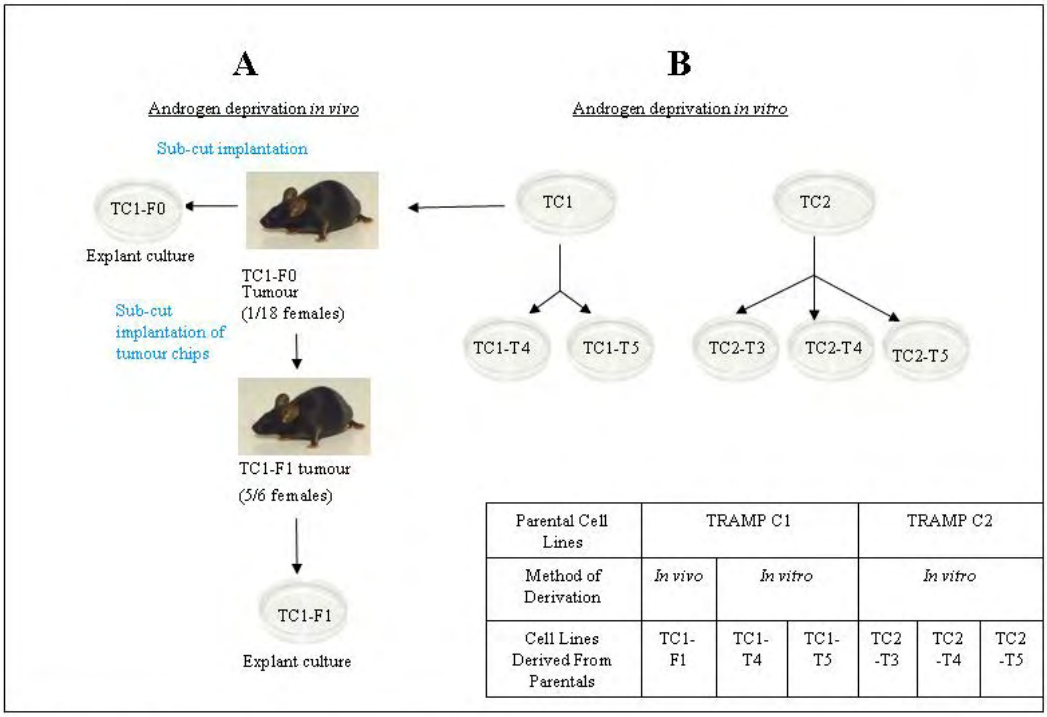
Although we didn't test these sublines *in vivo* for their metastatic potential, we chose metastases suppressor gene, KAI-1 for this study. To date, only a few metastasis-suppressor genes have been isolated from the human genome. KAI-1 is the first metastatic prostate cancer suppressor identified (33) with the Gleason grade and the clinical stage of PC showing an inverse relationship to KAI1 expression (34). It is also known as CD82 (C33 antigen), maps to 11p11.2 and encodes a transmembrane glycoprotein (35,36). Hence, it has been suggested that its loss may operate via enhancing the ability of cells to invade and metastasise via alteration of cell to cell interactions (37). KAI-1 has prognostic importance in advanced stage disease in different types of cancer including prostate (34,38), bladder (39), breast (40) and pancreas (41). The sublines showed variable but significant downregualtion of KAI-1 expression in comparison with the parental cells indicating that they are likely to have a greater metastatic potential than the parental cell lines and that they may represent different stages of the late stage disease.

The AI phenotype of the sublines was clearly established in our *in vitro* and *in vivo* studies. AR expression was clearly downregulated or mutated as shown by mRNA and protein expression studies. Interactions between androgens and AR maintain the essential equilibrium required for the normal function of the prostate gland with AR playing a pivotal role in pro-survival and proliferation during AI disease progression. It has been shown to be the major contributor towards the conversion of hormone responsive cancer to its AI phenotype. A number of mechanisms have been suggested to explain this including amplification of AR gene (42), mutations in the AR gene (43), and hypersensitivity to androgens (44), ligand independent activation of the AR from increased levels of co-activators and activation of alternative growth factor pathways (45). Our investigations at the mRNA and protein levels indicated that the AI phenotype of the new AI sublines cells may have been through selection of clones representing loss or downregulation of AR

at mRNA or through mutations in AR protein. At the transcriptional level (mRNA), AR downregulation in the sublines was significant but not completely absent. Interestingly, at the protein level, there was complete absence of AR in TC1-F1 and TC2-T5 cells/tumour, whilst the subline, TC1-T5 contained heterogeneous populations of AR-positive and -negative cells. This discrepancy in mRNA and protein expression in TC1-T5 may indicate the presence of mutated AR in these cells while in TC1-F1 and TC2-T5, the AR downregulation is likely to be at transcriptional level. While *in vitro* androgen-responsiveness of these cells was important for our study, we weren't able to test it due to the ban on the export of dihydrotestosterone from USA. Again when investigated for growth characteristics of these cell lines *in vivo* with or without androgen, the derivative subline tumors grew at the same rates with (sham castrated) and without androgen (castrated). The sublines were more aggressive as shown by two fold reduction in time taken to reach 15x15mm thershold. These data corelated with previous studies concerning the growth rate of TC1 and TC2 cell lines (9). Despite their considerably slower growth rates, the parental cells which are androgen-sensitive *in vitro*, showed some but not statistically significant differences in growth rates in castrated vs sham castrated mice. As discussed earlier, this may be because 1. of low tumourigenicity of ~95% androgen sensitive population in parental cell lines as reflected by low to none invasive ability and drastically reduced ability to form colonies in soft agar 2. <0.001% of highly tumourigenic AI cell population which takes over eventually, irrespective of the androgen status. This may explain the dramatically slow growth rates of the parental cell lines *in vivo* irrespective of the androgen status.

Hence, we conclude that the derivative sublines show androgen independence under the conditions tested in this study. The TRAMP model now encompasses androgen independent cell lines with different phenotypes and growth rates, representing a greater spectrum of the disease than previously. Further characterisation using focussed-array based gene expression profiling specifically for invasion and metastases related genes and assessment of *in vivo* metastatic ability of the sublines would contribute greatly towards better design and development of novel therapies for treatment of PC with greater clinical relevance.

Acknowledgements: We thank Sydney Foundation for Medical Research for providing funding for this project.



Parental Cell Lines	TRAMP C1			TRAMP C2		
Method of Derivation	*In vivo*	*In vitro*		*In vitro*		
Cell Lines Derived From Parentals	TC1-F1	TC1-T4	TC1-T5	TC2-T3	TC2-T4	TC2-T5

Figure 1. Derivation of androgen independent sublines from TRAMP C1 (TC1) and C2 (TC2) parental cell lines A Cell lines generated *in vitro* were grown in the absence of dihyhrotestosterone in charcoal dextran stripped foetal bovine serum (FBS). B Those generated *in vivo* were from TC1 cells injected subcutaneously into TRAMP female mice.

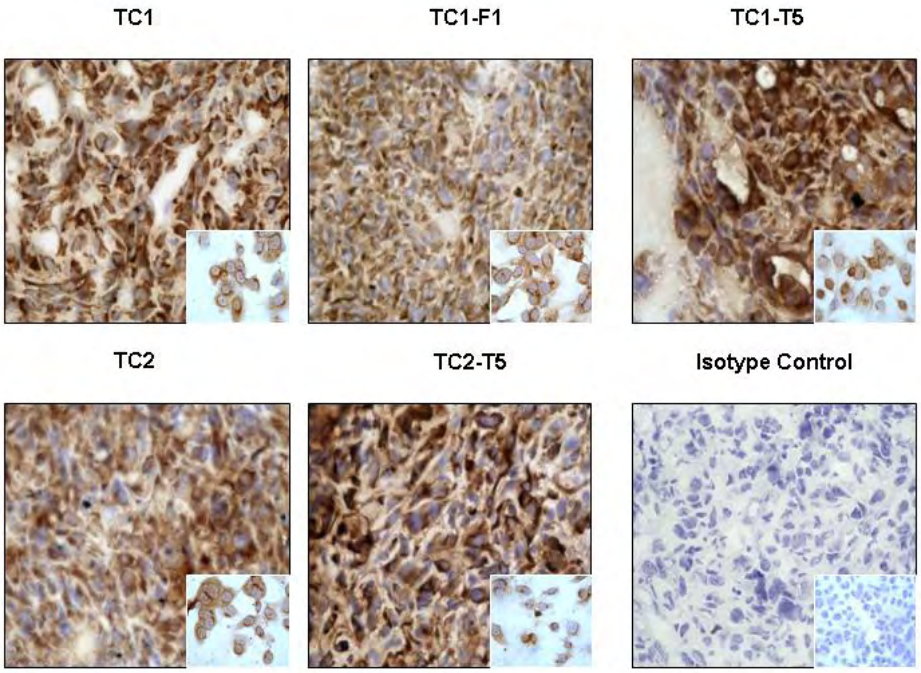


Fig.2- Pan Cytokeratin expression in different cell lines: Parental and derivative cell lines were immunostained for the epithelial marker, Pan-Cytokeratin. Immunohistochemical staining of frozen tumour sections and cultured cells grown on chamber slides (Inserts). Tissues and cells stained with isotype control were the negative controls. Magnification: X 40

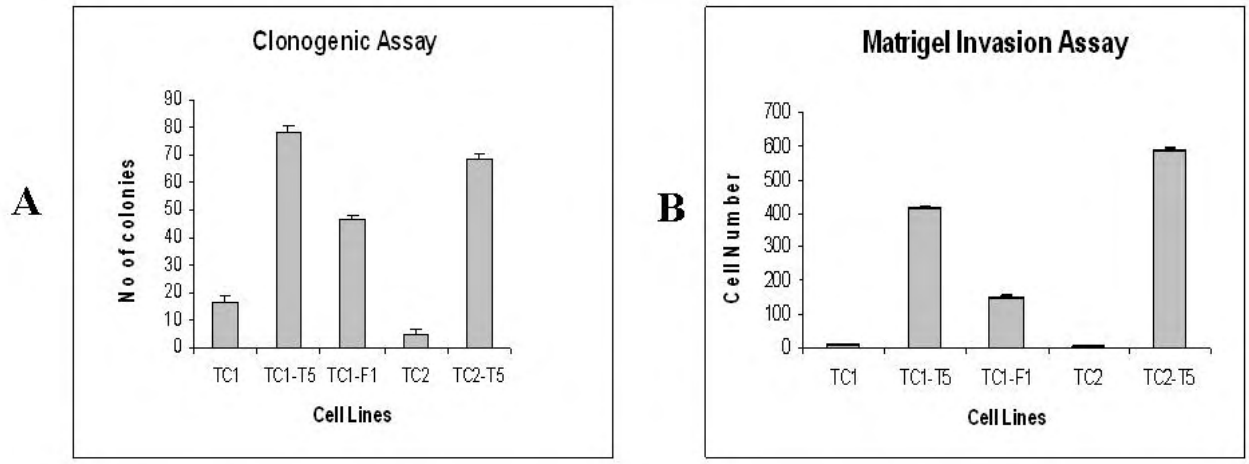


Fig. 3 Evaluation of anchorage- independent growth and invasiveness : Panel A Anchorage independent growth was determined by plating different cell lines in soft agar. Colony counts were performed 10 days later for new sublines and 14 days later for TC1 and TC2 lines (which grew more slowly). Panel B shows the number of cells (average number from 5 fields counted at x20) that invaded through matrigel (1:5 dilution) after 3 doubling times.

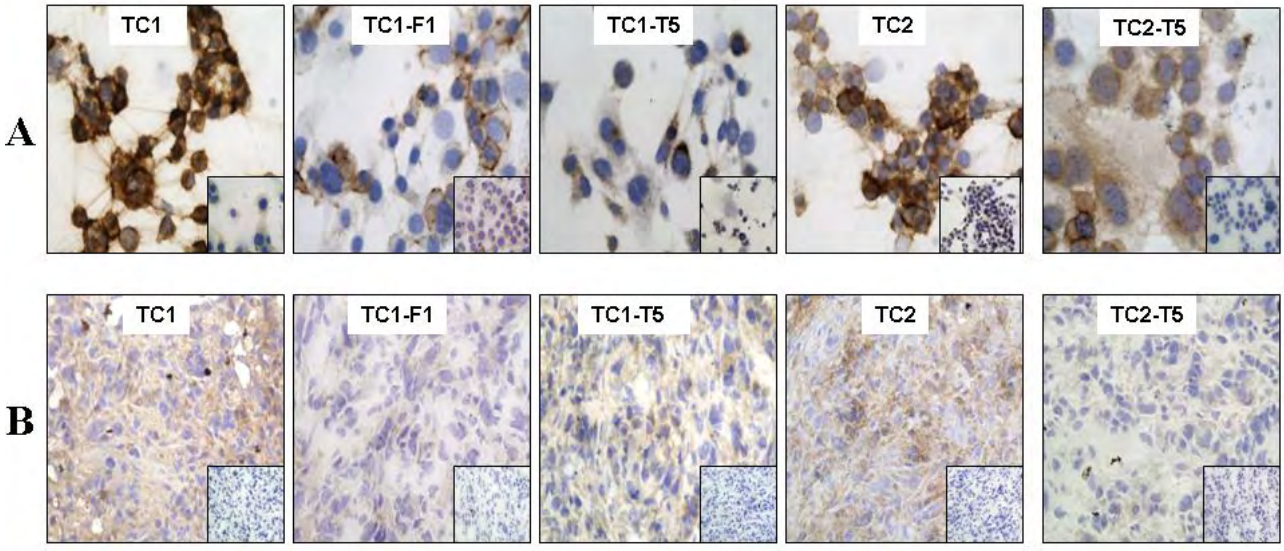


Fig.4- E-Cadherin and KAI-1 expression: Immunohistochemical analysis was performed for A, E-cadherin expression in cells grown in chamber slides *in vitro*, and B, KAI-1 expression in frozen sections from tumours grown *in vivo*. Inserts represent the respective isotype controls. Magnification X 20

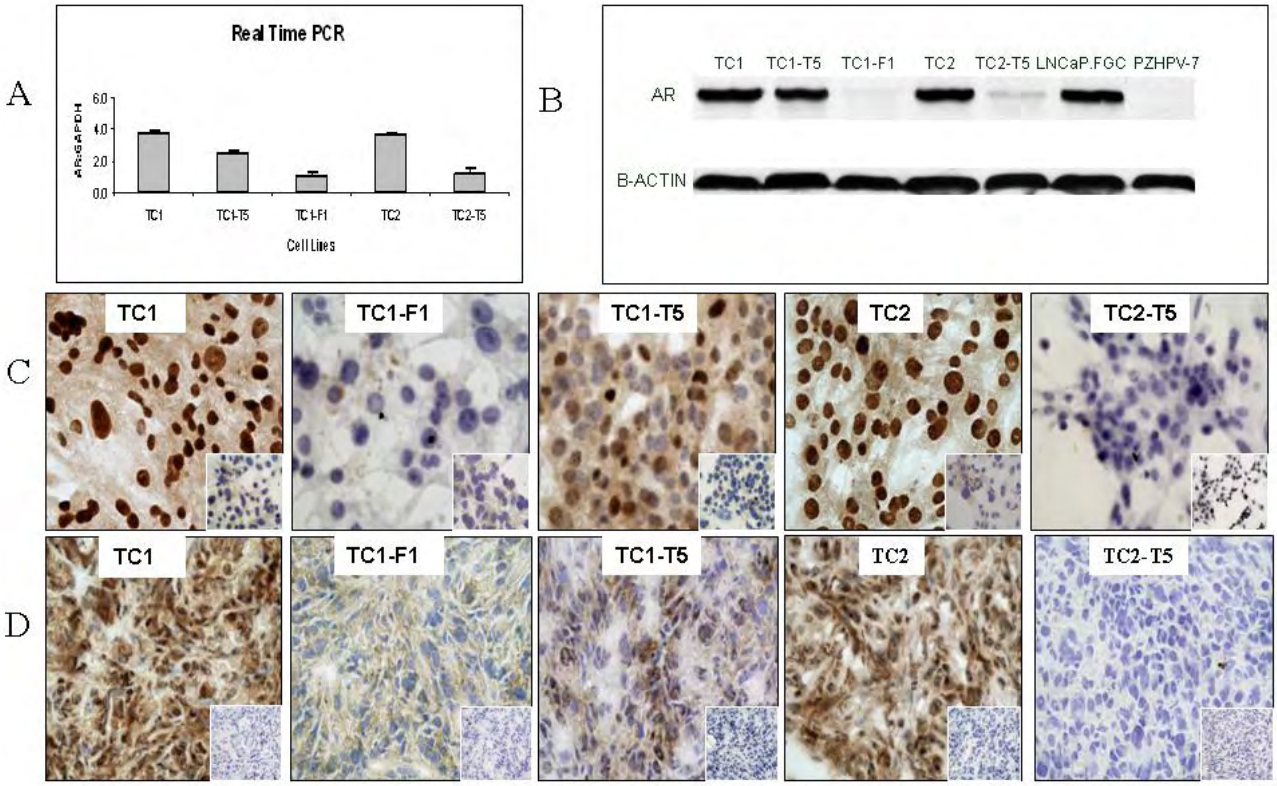
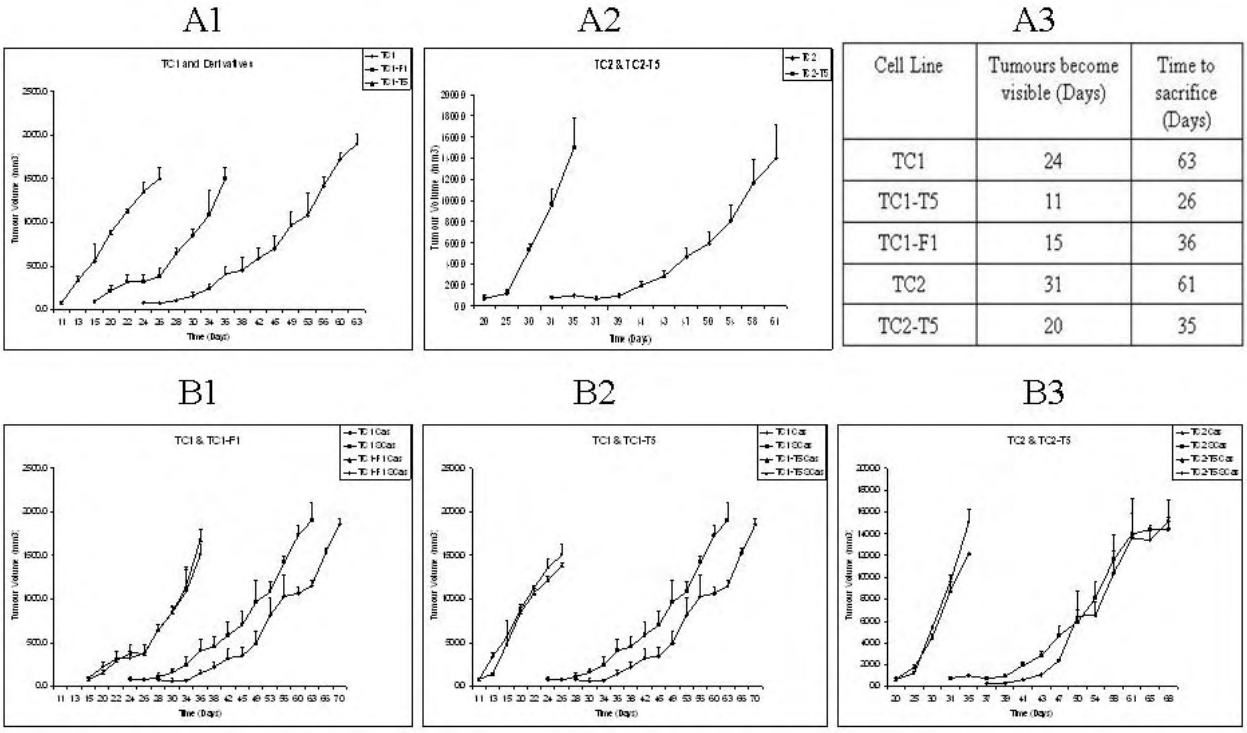


Fig.5- Evaluation of the Androgen Receptor status: *In vitro* AR expression was determined by real time PCR (Panel A), western blotting (Panel B) and immunostaining on chamber slides (Panel C). *In vivo* expression was assessed by immunohistochemistry (Panel D) on frozen tumour sections. Cells stained with the respective isotype control antibody (inserts) served as negative controls.



Cell Line	Tumours become visible (Days)	Time to sacrifice (Days)
TC1	24	63
TC1-T5	11	26
TC1-F1	15	36
TC2	31	61
TC2-T5	20	35

Fig 6- Androgen independent growth of TRAMP cell lines *in vivo*- Cells ($5x10^6$/100uL) were subcutaneously injected on the upper right hand flank of mice. For TRAMP C2 and TC2-T5, cells were mixed with matrigel (1:1)(100µL) before injection. Panel A(1-2): Tumour growth in male C57Bl/6 mice; Panel A3, numerical values.. Panel B (1-3): Graphic representation of tumour growth in castrated (Cas- triangles) and sham castrated mice (SCas- squares).

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Innovative Minds in Prostate Cancer Today (IMPaCT) submission-Atlanta, USA 2007

COMBINED GENE THERAPY WITH CYTOSINE DEAMINASE PLUS URACIL PHOSPHORIBOSYL TRANSFERASE AND IMMUNOSTIMULATORY IL12 AND IL18 CYTOKINES FOR TREATING PROSTATE CANCER IN C57BL/6 MICE.

Log #: PC001007

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Supported by US Army Medical Research and Material Command (Award:DAMD17-02-1-0107)

Running title: GDEPT plus cytokine therapy for prostate cancer

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Metastatic prostate cancer (PC) is hard to treat. We previously showed that using cytosine deaminase (CD) plus uracil phosphoribosyl transferase (UPRT) mediated gene directed enzyme prodrug therapy (CDUPRT-GDEPT) to treat mouse RM1 hormone-refractory prostate cancers (HRPC) in immunocompetent mice generated ***local and distant bystander effects*** with dose-dependent tumor infiltration by $CD4^+$ T cells, macrophages and NK cells (J Gene Med 2006;8:1086). As this suggested a potential for synergy with immunotherapy, using funding from a Department of Defense Prostate Cancer Award Alternative Funding grant, Fiscal year 2003 (DAMD17-02-1-0107), we tested the efficacy of CDUPRT-GDEPT together with immuno-stimulatory interleukins, murine (m) IL12 & IL18, against RM1 cells *in vivo*. Intraprostate (iprost) tumors of parental RM1 or stable transformants expressing GFP/CDUPRT (RM1CDUPRT) cells and RM1 lung pseudometastases were generated by iprost or intravenous (iv) injection respectively, in C57BL/6 mice. To assess cytokine effects, mice with iprost RM1 tumors were injected with Adenoviruses expressing mIL12 (AdmIL12) and/or mIL18 (AdmIL18) on day 5; on day 6, RM1 cells were given iv to establish lung metastases. Mice were euthanised on day 17, and prostate weight/volume, and lung colony counts assessed; tissues were assessed by immunohistochemistry and serum cytokine profiles by Luminex technology. To assess combined CDUPRT-GDEPT plus cytokine therapy, mice with iprost RM1CDUPRT tumors received AdmIL12 and/or AdmIL18 iprost on day 5, then 5-Fluorocytosine injections intraperitoneally for 11 days, and RM1 cells iv on day 6. As above, mice were euthanised for analysis on day 17; additionally, mouse survival was assessed.

Unlike mIL12 or mIL18 alone, their combination caused a significant reduction in local PC growth with clear synergy in reducing RM1 lung colonies. Serum cytokine analysis showed significant increases in Th1 IFN γ and IL18 and reduction in Th2 IL4 and IL10 levels in the mIL12 + mIL18 group. Combining CDUPRT-GDEPT with mIL12 + mIL18 led to further growth reduction of local PCs and lung colonies compared with individual therapies. There was a clear survival advantage with 28.7% of mice alive on day 33, despite suppression of serum cytokine levels compared with controls. We conclude that combined CDUPRT-GDEPT with cytokine therapy provides a significant reduction in growth of local and remote deposits of HRPC RM1 tumors in mice. Despite their immunosuppressed state, combination therapy was more effective and provided survival advantage.

IMPACT: This project has the potential to impact on PC mortality through the generation of local and anti-metastatic effects by local delivery of combined GDEPT and cytokine gene therapy. Such therapy may well improve the prognosis for PC for those thousands of men who succumb to the disease each year.

Combined Gene Therapy with Cytosine Deaminase plus Uracil Phosphoribosyl Transferase and Immunostimulatory IL12 and IL18 Cytokines for Treating Prostate Cancer in C57BL/6 mice.

Abstract#975

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Background

Gene-directed enzyme prodrug therapy (GDEPT) based on a fusion gene from *E.coli,* cytosine deaminase uracil phosphoribosyl transferase (CDUPRT), provides a new approach to the treatment of slow growing tumors like prostate cancer (PC). We previously found ***local & distant bystander effects using*** CDUPRT-GDEPT to treat mouse RM1 hormone-refractory prostate cancers (HRPC) in immunocompetent C57BL/6 mice. Treatment induced dose-dependent tumor infiltration by CD4+ T cells, macrophages and NK cells, suggesting potential for synergy with immunotherapy. In this study,, we have evaluated the therapeutic potential of combining immuno-stimulatory interleukins IL12, IL18 of proven anti-tumorigenic potential (enhanced when combined) with CDUPRT-GDEPT for treating HRPC in immunocompetent mice.

Aim

To test the efficacy of combining CDUPRT-GDEPT with mIL12 + mIL18 cytokine treatment against mouse HRPC RM1 cells *in vivo* in an orthotopic immune competent mouse model.

Methods

HRPC mouse RM1 cells or those stably transformed to express GFP/CDUPRT (RM1CDUPRT) were used to generate orthotopic RM1 or RM1CDUPRT tumors or RM1 lung pseudometastases in C57BL/6 mice.

To assess the effects of the cytokine therapy (n=10), Intraprostatic RM1 tumors (4x4mm) in mice were injected with $2x10^7$plaque forming units (pfu) of replication defective Adenoviruses expressing murine IL12 (AdmIL12) and/or murine IL18 (AdmIL18)(day 5); on day 6, RM1 cells ($2.5x10^5$)were given intravenously (iv) to establish lung pseudometastases. Mice were euthanased on day 17 and the following analyses were carried out:

•**Evaluation of tumor growth and lung colony formation**: Harvested PCs were weighed and measured using vernier calipers across two diameters, d1 and d2, at right angles . The tumor volume (V) was calculated: $V = \pi/6(d1 \times d2)^{3/2}$. Harvested lungs were fixed with Bouin's solution to visualise lung lesions (1x1 mm) using a dissecting microscope

•**Immunostaining and serum analyses**: Tumors, lymph nodes, spleens and lungs were frozen or formalin fixed for immunohistochemistry. 5 micron paraffin sections were stained for apoptosis and proliferating cells by *in situ* detection using M-30Cytodeath™ (Alexis Biochemicals) or Ki-67(BD-Pharmingen) antibodies respectively. Acetone-fixed fresh frozen sections were stained for infiltrating T lymphocytes (rat anti-mouse CD4, BD-PharMingen 1:100; rat antimouse CD8a (Ly-2) BD-PharMingen, 1:200); macrophages (rat antimouse F4/80, BD-PharMingen, 1:800) and NK cells (rabbit antimouse AsialoGM1 (Wako) 1:400, The mean number of stained cells/high power field was averaged over 10 fields. Mouse sera were analyzed (n=5) using *Luminex technology* according to the recommended protocol to assess cytokine profiles.

Combination of CDUPRT-GDEPT with cytokine therapy (n=10), Intraprostatic RM1CDUPRT tumours (4x4mm) were given AdmIL12+AdmIL18 at $2x10^7$ pfu (day 5). A day later, 5FC was given (ip) for 11 consecutive days; RM1 cells were given iv to establish lung colonies. The protocol at necropsy was as above. Survival of mice in different treatment groups was also assessed.

Results

1. Combination of mIL12 and mIL18 therapy led to a significant reduction in growth of intraprostatic RM1 tumors and in RM1 lung psueodometastases in C57 BL/ 6 mice

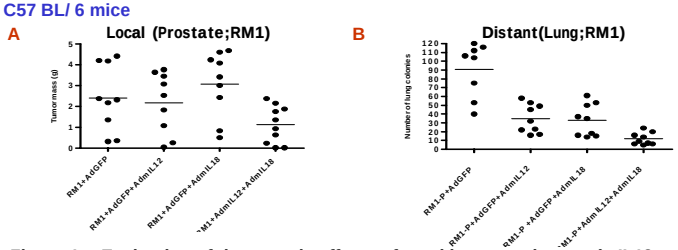

Figure 1: : Evaluation of therapeutic effects of cytokine gene therapy (mIL12 or mIL18 or both) in treatment of RM1 Intraprostatic tumours and lung pseudometastasis: (**A**) Graphical representation of tumour growth in the prostate using different treatments. $5x10^3$ RM1 cells were implanted Iprost in C57BL/6 mice (n=10). 5 days later, cytokines (AdmIL12 and/or AdmIL18 or AdGFP) were given intratumourally (i.t). At necropsy (day17), prostate tumor volume/mass was measured. **(B)** Graphical representation of number of lung colonies using different treatments. $5x10^3$ RM1 cells were implanted Iprost in C57BL/6 mice (n=10). 5 days later, cytokines (AdmIL12 and/or AdmIL18) saline were given i.t. At necropsy (day 17), lung colony count was done as described in methods.

2. Combining CDUPRT-GDEPT with mIL12 + mIL18 therapy led to a further reduction in growth of both local PCs and lung colonies compared with individual treatments and a significant survival advantage .

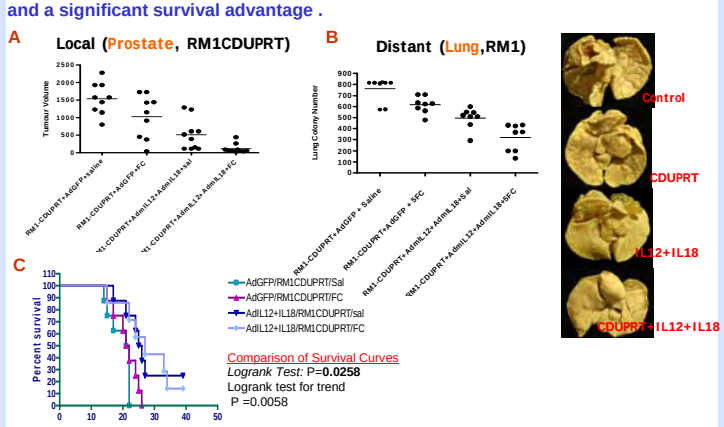

Figure 2: : Evaluation of therapeutic effects of combining cytokine gene therapy with CDUPRT-GDEPT in vivo. RM1GFPCDUPRT cells ($5x10^3$) were implanted Iprost in C57BL/6 mice (n=10). Day 5: i.t. administration of AdmIL12 and AdmIL18 or AdGFP Day 6: iv implantation of RM1 cells ($2.5x10^5$), IP 5FC administration daily for 11 days until necropsy (day 17) **(A)** Graphical representation of tumor growth in the prostate using different treatments. **(B)** Graphical representation of number of lung colonies using different treatments. Photographs of representative samples from different groups are shown. **(C)** Survival curves of tumor bearing mice undergoing different treatments (n=10/group).

Results

3. CDUPRT-GDEPT+ cytokine therapy led to substantial enhancement of tumour infiltration by macrophages, $CD4^+$T, $CD8^+$T and NK cells. The mechanism also may involve disruption of tumour vasculature and reduction in cell proliferation.

Table 1: Immunohistochemical evaluation of tumor infiltration with immune cells, extent of apoptosis, proliferation and vasculature disruption.

Treatment groups	*Immunohistochemical scores[1] ± SEM*						
	T helper cells ($CD4^+$)	*Cytotoxic T Lymphocyte ($CD8a^+$)*	*NK cells (Asialo-GM^+)*	*Macrophages ($F4/80^+$)*	*Endothelial cells ($CD31^+$)*	*Proliferation ($Ki\text{-}67^+$)*	*Apoptosis ($M30^+$)*
RM1CDUPRT/Saline AdGFP	**16.3±2.6**	**23.6±7.0**	**7.2±2.4**	**36.9±3.0**	**100.4±19.3**	**187.4±12.4**	**1.7±0.3**
RM1CDUPRT/5FC AdGFP	**17.6±3.5**	**29.8±8.8**	**3.1±1.4**	**25.9±6.3**	**66.4±6.1**	**101.1±5.8**	**3.0±0.9**
RM1CDUPRT/Saline AdmIL12/AdmIL18	**28.4±5.7**	**41.0±8.5**	**25.6±7.9**	**74.6±7.7**	**57.9±4.8**	**160.0±6.1**	**38.1±1.5**
RM1CDUPRT/5FC AdmIL12/AdmIL18	**80.7±18.4**	**69.4±13.4**	**81.0±6.3**	**173.8±17.1**	**21.4±3.6**	**64.3±20.4**	**1.3*±0.14**

[1]Scoring was done in 10 high power fields/mouse (n=4)

4. CDUPRT-GDEPT +Cytokine therapy led to a general skewing towards Th1 type immune responses.

Table 2: Serum Cytokine levels in response to different treatments on day 17

Cytokines tested	***GDEPT***	***Cytokines***			***GDEPT+ IL12+IL18***
		IL12	***IL18***	***IL12+IL18***	
IL-2	**U***	**D**	**U***	**D**	**U***
IL-4	**D***	**D***	**D****	**D*****	**D****
IL-5	**U*****	**D**	**D**	**D/-**	**U****
IL-10	**-***	**D***	**D****	**D*****	**D***
IL-12	**-**	**-***	**-***	**D/-**	**-**
TNF-A	**U***	**D***	**D****	**D**	**U***
IFN-G	**D**	**-**	**U***	**U****	**D**
GMCSF	**U****	**-***	**-***	**-***	**U****
RANTES	**D***	**-***	**-***	**D***	**U****
MCP-1	**D***	**D****	**D***	**D****	**D****
*IL18**	**D***	**U***	**U*****	**U****	**D****

Serum cytokine analysis (Luminex) showed a significant increase in Th1 cytokine (IL2, TNF-A, and GMCSF) and chemokine (RANTES) and a significant reduction in Th2 cytokine (IL10 and IL4) and chemokine (MCP-1) levels.

U: Upregulation Vs control group (RM1CDUPRT/AdGFP/Saline);D: Downregulation Vs control group (RM1CDUPRT/AdGFP/Saline); * Values > reference value from healthy C57BL/6 sera. Number of * correlate with the relative values within the group

Conclusions

•Combined CDUPRT-GDEPT with mIL12+mIL18 therapy led to a significant reduction in growth of local and remote deposits of HRPC RM1 tumors in mice and offered a survival advantage to the treated mice.

•The antitumour activity was associated with low tumor cell proliferation in the prostate and infiltration of the treated prostate by CD4+ T cells, macrophages and NK cells. Production of Th1 type of cytokine/chemokines may have led to the improved outcome observed with the combination treatment.

Acknowledgements

The work was supported by Department of Defense, US Army,Prostate Cancer grant number: DAMD-17-1-02-0107.